LUIS F. LELOIR

PREMIO NOBEL DE QUIMICA 1970

OPERA SELECTA

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PRESENTACION

En 1970 la Real Academia de Ciencias de Suecia otorgó al Dr. LUIS FEDERICO LELOIR el premio Nobel de Química por su "descubrimiento de los nucleótido-azúcares y su función en la biosíntesis de los hidratos de carbono".

La Academia Nacional de Ciencias Exactas Físicas y Naturales, el Consejo Nacional de Investigaciones Científicas y Técnicas y la Facultad de Ciencias Exactas y Naturales, instituciones a las que pertenece el laureado, resolvieron tributar un homenaje de reconocimiento a quien, con su reconocido esfuerzo y talento, proporcionó al país un lauro, motivo de legítimo orgullo para la ciencia argentina.

Por ello, de común acuerdo, decidieron efectuar esta publicación de sus trabajos más importantes, homenaje que pone de relieve, en forma perdurable, el origen de tan merecido galardón.

Los editores de esta selección de trabajos agradecen a las siguientes Instituciones y Editoriales, la autorización que le han concedido para la reproducción de los artículos y trabajos originales que se mencionan en cada caso:

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* Fallecido el 7 de marzo de 1973.
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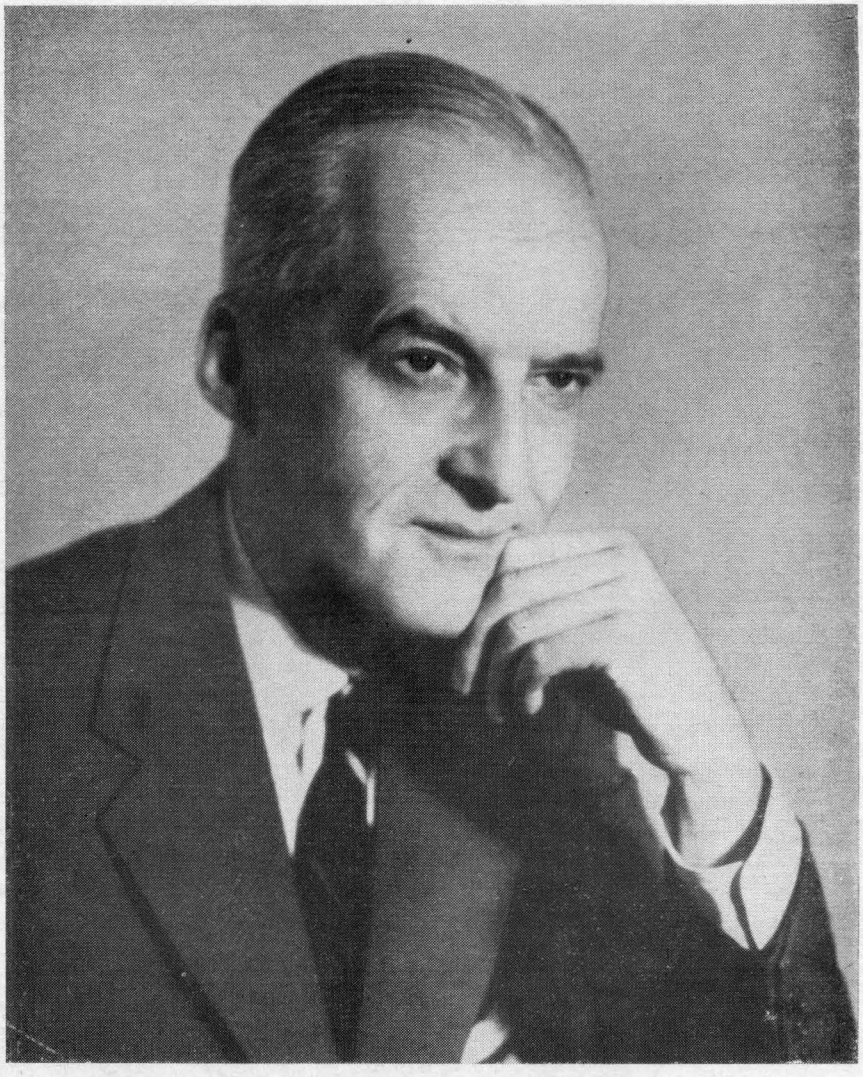


Louis Rheiner

DOCTOR LUIS F. LELOIR, EL INVESTIGADOR *

por el Académico Titular Dr. VENANCIO DEULOFEU

Conocí al Dr. Luis Federico Leloir en el año 1932, cuando comenzó a trabajar en el Instituto de Fisiología de la Facultad de Medicina, que dirigía el Dr. Bernardo A. Houssay. Tuvo su primer lugar de trabajo en el más grande de los recintos que existían en el sótano del Instituto, cuyo moblaje principal y más lujoso, lo constituían una media docena de mesas con tapa de lava, espléndida esta última, y que si no fuera por su tamaño serían hoy piezas de colección. Cada investigador podía disponer de una mesa, y a veces de un anexo que era otra mesa, esta vez de azulejos, adosada a una ventana, con un pequeño armarito de madera en un extremo.

El laboratorio contenía un aparato importante, una centrífuga, en la cual podían colocarse tubos de 250 ml. y otros aparatos menos importantes, por su precio, como los colorímetros de Dubosq, o los aparatos de van Slyke para determinar reserva alcalina. La limpieza del material debía ser realizada por quien lo empleaba, en especial aquellos que recién se iniciaban. Con el tiempo podía buscarse quien se ocupara de hacerlo, si se disponía de ciertas dotes y medios de convicción. La limpieza tenía menos importancia que actualmente, pues afortunadamente tanto la cromatografía en papel como en placa fina no existían, lo cual permitían calificar de "puras" a muchas substancias que hoy no lo serían. Por otra parte, fuera de las drogas más empleadas, las restantes debían ser preparadas por los interesados La investigación bioquímica y biológica, no había aún llegado a ser "big business".

En ese ambiente, se inició Leloir en la investigación científica. Se había recibido de médico, y asistido a algunos servicios clínicos en los hospitales Ramos Mejía y de Clínicas, donde había escuchado comentarios favorables sobre los trabajos que se efectuaban en el Instituto. No hacía mucho tiempo que Houssay había dado a conocer una serie de investigaciones de mucha importancia, efectuadas en parte en colaboración con el Dr. Alfredo Biasotti, demostrando el papel de la hipófisis en el metabolismo de los hidratos de carbono. El Dr. Carlos Bonorino Udaondo, a cuyo servicio del Hospital de Clínicas concurría el Dr. Leloir, lo presentó al Dr. Houssay para que le diera un tema de tesis, que realizaría en el Instituto. El tema elegido consistió en estudiar el papel de las suprarrenales en el metabolismo de los azúcares.

En realidad, el ambiente físico donde se trabajaba estaba ampliamente compensado por la calidad del Director del Instituto, a quien se habían sumado, atraídos por su prestigio, un buen número de personas que iba en aumento, interesadas en iniciarse en la investigación biológica y biomédica y que concurrían a trabajar en forma continua y sistemática. Se había formado un grupo con interés general en favor del progreso de la ciencia argentina, pues consideraba que su desarrollo era de importancia para el adelanto futuro del país.

Leloir trabajaba en forma disciplinada. Llegaba y partía a horas casi fijas. Dedicado a su labor, conversaba poco, almorzaba frugalmente en el Laboratorio y solía descansar fumando un cigarrillo.

Los resultados que obtuvo le permitieron presentar su tesis de Doctorado con el título de "Suprarrenales y metabolismo de los hidratos de carbono" que mereció el Premio Facultad del año 1934. Entre 1933 y 1935 participó junto con Houssay, Novelli, Dambrosi, Foglia, del Castillo y Fernández, en una serie de publicaciones vinculadas con ese tema, que aparecieron en la *Revista de la Sociedad Argentina de Biología* y en los *Comptes Rendus de la Societé de Biologie de Paris*.

Durante esos años Leloir vivió en un ambiente donde se desarrollaban varias líneas de investigación. Conoció y trató a quienes trabajaban en ellas, interesándose en las mismas, en las metódicas y en los equipos que se

* Del Boletín de la Academia Nacional de Medicina. Vol. 50, p. 6, 1972.

empleaban. Es evidente que en esa época comenzó a interesarse en la bioquímica, que pensaba que podía aclarar los mecanismos íntimos de los procesos fisiológicos.

Cuando el Dr. Houssay le aconsejó que pasara un tiempo en un laboratorio extranjero, la decisión fue elegir un lugar donde se cultivara la bioquímica. Se eligió uno de los mejores, el de la Universidad de Cambridge, cuyo Director, Frederick Gowland Hopkins, es hoy una figura legendaria de la época inicial de las investigaciones bioquímicas, a quien se debió la apertura de nuevas ramas de las mismas.

Leloir había adquirido una personalidad definida durante los años que había permanecido en el Instituto. Al despedirlo con motivo de su partida a Inglaterra pude decirle: "Constituye un raro ejemplo de interés por los más diversos temas científicos. No conozco capítulo alguno de las ciencias físico-químicas y biológicas que no le preocupe. Desde la estructura del núcleo atómico hasta los principios de la termodinámica, pasando por el cálculo diferencial y desde la fisiología de las suprarrenales hasta la radiación mitogenética, a través de los problemas de la respiración celular o del quimismo muscular; todo le llama la atención y le atrae. Lee sobre todos los temas y pregunta también sobre todos. Y esto, que a la edad adulta puede ser inconveniente, resulta en la suya una ventaja. Y como tiene receptividad para los nuevos conocimientos, no me cabe la menor duda que ha de aprovechar su estada en Cambridge en forma debida".

En Cambridge, Leloir se inició en las técnicas enzimáticas trabajando con Edson, Dixon y David E. Green. Volvió de Inglaterra no sólo con nuevos conocimientos, sino con la experiencia de haber vivido en una escuela destacada por sus investigaciones. Trajo consigo una buena parte del equipo para poder iniciar investigaciones sobre enzimología en el Instituto de Fisiología. Dispuso de todo un laboratorio, relativamente amplio, que luego se fue expandiendo, pero el habitat fue siempre el sótano del Instituto.

Allí comenzó sus nuevos trabajos. Con J. M. Muñoz, quien se había interesado en el tema, estudiaron la oxidación enzimática del alcohol y posteriormente la oxidación de los ácidos grasos por enzimas del hígado, describiendo por vez primera una preparación libre de células capaz de producirla.

Poco después, Leloir y Muñoz se integraron al grupo formado por Eduardo Braun Menéndez, Juan Carlos Fasciolo y Alberto C. Taquini, que investigó y aclaró el mecanismo de la hipertensión arterial de origen nefrógeno. Demostraron que la substancia responsable de esa hipertensión era una proteína producida en el riñón, la renina, que actuando sobre otra substancia proteica de la sangre, determinaba la producción de polipéptidos hipertensores, actualmente llamados angiotensinas (al comienzo le dieron el nombre de hipertensina).

Eduardo Braun Menéndez me ha contado en más de una ocasión, cómo Leloir señalaba que la formación de hipertensina tenía todos los aspectos de deberse a una reacción enzimática. Cuando se encontraron las condiciones adecuadas, pudieron demostrar que la renina actuaba como una enzima, frente a un substrato proteico existente en el suero. Los resultados de estas investigaciones fueron condensados en un libro que con el nombre de "Hipertensión Arterial Nefrógena", se publicó en 1943 y obtuvo el tercer Premio Nacional de Ciencias correspondiente al trienio 1942-1944. Fue posteriormente traducido al inglés y al italiano.

En ese año Leloir casó con Amelia Zuberbühler, quien lo ha acompañado y se ha interesado siempre por sus trabajos y con una gran comprensión por la labor que realiza, lo ha estimulado, apoyándolo continuamente en su quehacer diario.

Viajaron ese mismo año a los Estados Unidos, donde Leloir trabajó primero en el Laboratorio de Carl F. Cori, en la Universidad de Washington, en San Luis, y luego se trasladó a la Universidad de Columbia en Nueva York, donde se unió nuevamente con David E. Green, su antiguo compañero de tareas en Cambridge.

A su regreso volvió al Instituto de Fisiología, nuevamente dirigido por Houssay, quien había sido obligado a dejar el cargo por un tiempo. En esa época retornó al país Ranwel Caputto, que se había iniciado en los estudios enzimológicos en Córdoba junto con Alberto Marsal y quien como becado de la Asociación Argentina para el Progreso de las Ciencias había trabajado en Cambridge, en el mismo laboratorio que Leloir. Caputto recibió una beca interna especial de la Asociación y se asoció con Leloir, iniciando una investigación sobre la síntesis de la lactosa en la glán-

dula mamaria. El problema presentó dificultades que los indujeron a estudiar el mecanismo de la fermentación de la galactosa en microorganismos, lo que determinó la incorporación de Raúl E. Trucco a esos trabajos. Fue en esa época cuando Jaime Compomar ofreció al Dr. Bernardo A. Houssay crear con su esposa, en memoria de sus padres, un laboratorio de investigaciones bioquímicas. Casi al mismo tiempo, Houssay debió alejarse nuevamente de la Facultad y volvió a dedicarse al Instituto de Medicina y Biología Experimental, fundado por acción de un grupo de personas, para que pudiera continuar investigando, cuando se había producido el episodio de su primer retiro. Al mismo tiempo se trasladaron también Leloir, Caputto y Trucco hasta que pudieron alquilar una casa vecina. En esa casa tuvo lugar la inauguración formal, en 1947, del Instituto de Investigaciones Bioquímicas Fundación Campomar. El grupo de trabajo se había aumentado con la incorporación de Carlos E. Cardini y de Alejandro C. Paladini.

Fue en ese lugar donde se efectuaron importantes hallazgos que abrieron nuevos rumbos al conocimiento del metabolismo molecular de los hidratos de carbono. Los mismos no fueron determinados por un cambio de tema sino por el contrario, en el empeño de continuar estudiando el metabolismo de la lactosa, esta vez empleando un microorganismo, el *Saccharomyces fragilis*. En 1948, en un trabajo firmado por Leloir, Trucco, Caputto y Mittelman, dan cuenta de la presencia en dicha levadura de una enzima que cataliza la formación de galactosa-1-fosfato a partir de galactosa y de ATP. Encontraron que esta sustancia se metaboliza de acuerdo a las siguientes transformaciones:

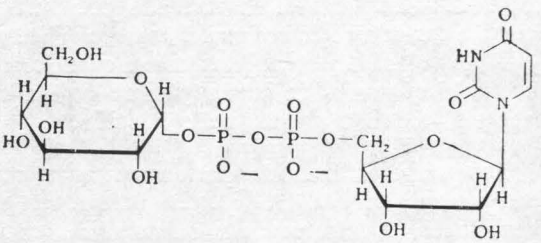
$$\text{Galactosa-1-fosfato} \rightleftarrows \text{Glucosa-1-fosfato} \rightleftarrows \text{Glucosa-6-fosfato} \qquad (1)$$

La transformación de la glucosa-1-fosfato en su isómero la glucosa-6-fosfato, catalizada por la fosfoglucomutasa, necesitaba un factor termoestable (coenzima) para que la enzima pudiera ejercer su acción.

En muy poco tiempo aislaron dicho factor y determinaron que era la glucosa-1,6-difosfato. Ha recibido en la literatura el nombre de ester de Leloir y fue la primera coenzima que se aisló en el laboratorio y en el país.

La identificación de la coenzima de la fosfoglucomatasa fue un trabajo de la mejor calidad, que no aclaró sin embargo el problema de la transformación de la galactosa-1-fosfato en glucosa-1-fosfato, vale decir de la primera de las dos etapas indicadas en la ecuación 1.

Profundizando el estudio, encontraron también que para esa primera etapa era necesario un factor termoestable. En 1949 pudieron demostrar esa necesidad y un año después describir su purificación e identificación. Era un dinucleótido con una estructura que se encontraba por vez primera en los organismos. Por esta razón y por la importancia que su hallazgo ha tenido, consideramos oportuno indicarla.



Está formado por la condensación del ácido uridílico con la glucosa-1-fosfato por medio de uniones fosfóricas y se lo denomina uridina-difosfo-glucosa (= UDPG) . Resultó necesario para que pueda actuar la enzima que cataliza la transformación de la glucosa en galactosa, la cual se produce sin que ninguno de los dos azúcares se libere del dinucleóxido, obteniéndose uridina-difosfo-galactosa (UDP-Gal) .

Esta reacción fue estudiada con mucho detalle por Leloir (1951) quien demostró, empleando la enzima de *S. fragilis*, que es reversible:

$$\text{UDPG} \rightleftarrows \text{UDPGal}$$

y que en el equilibrio, un 25 % es UDPGal.

Leloir escribió las reacciones de transformación en la siguiente forma:

$$\text{Galactosa-1-fosfato} + \text{UDP-glucosa} \rightleftarrows \text{Glucosa-1-fosfato} + \text{UDP-D-galactosa} \qquad (2)$$

$$\text{DDP-D-galactosa} \rightleftarrows \text{UDP-D-glucosa}$$

las cuales sumadas indican la transformación que ocurre

$$\text{Galactosa-1-fosfato} \rightleftarrows \text{Glucosa-1-fosfato}$$

Esta coenzima, la UDP-glucosa, adquirió con el tiempo importancia considerable, porque se demostró que su función no estaba restringida a la interconversión de los monosacáridos mencionados, sino porque actuaba en muchas otras reacciones de importancia metabólica. Además señaló el comienzo del hallazgo en los organismos animales y vegetales de una larga lista de factores co-enzimáticos de estructura similar, en los cuales la base piridínica que la forma, el uracilo, puede estar sustituida por otras que pueden ser tanto de la serie pirimidínica como de la serie de las purinas, al igual que la glucosa o la galactosa pueden ser reemplazadas no sólo por otros monosacáridos sino también por sustancias de estructura más simple, como el glicerol o el ribitol o más complejas como cuando son sustituidas por el ácido murámico que a su vez está unido a un pequeño péptido.

Los primeros estudios que demostraron que la glucosa del UDPG podía intervenir en reacciones de transferencia, cuando se encuentra el aceptor adecuado, fueron realizados en el laboratorio de Leloir. Con Cabib demostraron (1953), que los extractos de levadura contienen una enzima que cataliza la trasferencia de la glucosa a la glucosa-6-fosfato que actúa como receptor, formándose trehalosa fosfato, ester fosfórico del disacárido trehalosa. Poco después Leloir y Cardini (1953-1955) demostraron que cuando el aceptor es la fructosa o la fructosa-6-fosfato, se forman respectivamente sacarosa o sacarosa-fosfato, mecanismo que produce uno de los disacáridos más distribuidos en los vegetales y posiblemente el producto natural que se produce industrialmente, con elevada pureza, en mayor cantidad. Simultáneamente aislaron algunas nuevas coenzimas de dicho grupo. Cabib, Leloir y Cardini (1953) obtuvieron la UDP-N-acetil-glucosamina, Cabib y Leloir (1954) la guanosin-difosfato-manosa y Pontis (1955) la UDP-N-acetil-galactosamina.

La importancia de las reacciones en que estos dinucleótidos intervenían produjo una expansión de sus estudios, que se iniciaron en *muchos laboratorios* y la lista de los que se han obtenido y de sus funciones es impresionante.

En estos momentos en que se discute mucho en todo el mundo sobre el apoyo que debe prestarse a la ciencia pura, o a la investigación por la investigación misma, conviene señalar que los hallazgos de Leloir y colaboradores, cuyos trabajos no creo que estuvieron motivados por una posible utilidad futura, han producido desde la primera época una ampliación en el conocimiento de más de un problema de naturaleza médica. Pueden darse dos ejemplos: los estudios de Kalckar han demostrado que la galactosemia de los niños se debe a la disminución o carencia congénita de la enzima que cataliza la transferencia indicada en la ecuación (2) lo que determina la acumulación de galactosa-1-fosfato y de galactosa en el organismo; otro ejemplo ha sido la demostración que la transferencia de ácido glucurónico en los organismos, a sustratos normales, como la bilirrubina, para formar el producto conjugado o bien a sustratos extraños, como los alcoholes y los fenoles, en los procesos llamados de desintoxicación, se realiza a partir de UDP-ácido glucurónico, que a su vez se forma por oxidación de la glucosa que forma parte del UDPG.

Si se piensa que ahora se conoce que estas coenzimas intervienen en la formación de glicósidos animales y vegetales, en la formación de polisacáridos como el almidón y el glucógeno, en la biosíntesis de los ácidos hialurónicos, y de sustancias que forman parte de las paredes celulares, se tiene un panorama de la importancia del hallazgo del UDPG, que parecía ser necesaria para catalizar una reacción solamente de interés en las levaduras o para la formación de lactosa en los mamíferos.

En medio de toda esta expansión en los estudios de los dinucleótidos que actúan como coenzimas, Leloir ha permanecido fiel a los hidratos de carbono, interesándose por la biosíntesis de polisacáridos de alto peso molecular, como el glucógeno y el almidón.

Ya en 1957 habían encontrado con Cardini que en el hígado y el músculo existían enzimas capaces de transferir glucosa del UDPG al glucógeno existente en esos órganos. A partir de este hallazgo ha desarrollado una serie de trabajos. Su principal deseo era obtener *in vitro* partículas de glucógeno que resultaran idénticas en su comportamiento a las que pueden aislarse de los tejidos.

En diversas investigaciones (1965-1969) en las cuales han participado varios de sus colaboradores (Krisman, Mordoh, Parodi) lograron tener éxito cuando emplearon como dador de glucosa para la formación del polisacárido, al UDPG, y como enzima la glucógeno sistetasa del hígado. El glucógeno es un polisacárido ramificado y el sistema debía

completarse con la adición de la llamada enzima ramificante, que precisamente cataliza la transferencia, dentro de la misma molécula, de cadenas cortas, lineales, formadas por unidades de glucosa, en forma tal que se creá una estructura ramificada.

El glucógeno así obtenido era de peso molecular elevado, del orden del que posee el glucógeno natural, y se comportaba como éste frente a la acción del calor, de los ácidos o de los álcalis. No ocurrió lo mismo cuando se preparó glucógeno empleando glucosa-1-fosfato como substrato y fosforilasa como enzima, además de la enzima ramificante. Se obtienen también productos de alto peso molecular, pero sus propiedades no resultan del todo idénticas al natural.

Al ampliar estos estudios a la formación del almidón, Fekete, Leloir y Cardini (1960-1961) encontraron que los granos de este polisacárido contienen una enzima capaz de catalizar la transferencia de glucosa del UDPG no sólo a los granos de almidón, sino también a otros aceptores de bajo peso molecular, formados por pocas moléculas de glucosa. La sustitución del UDPG como dador de glucosa por otras coenzimas de este grupo, permitió comprobar que la adenosina-difosfo glucosa (ADPG) era más activa que la primera en la reacción de transferencia (Recondo y Leloir, 1961). Estos trabajos han sido continuados en el laboratorio particularmente por Carlos E. Cardini y sus colaboradores.

Las investigaciones sobre la participación de estas coenzimas en la formación de polisacáridos han introducido a Leloir al estudio de la función de los lípidos en la transferencia de monosacáridos en los tejidos animales. Uno de estos lípidos es el monofosfato de dolicol (DMP, ester monofosfórico de un alcohol primario, formado por unos 20 residuos isoprénicos.

Han encontrado que los microsomas hepáticos contienen enzimas que catalizan la transferencia de glucosa de la UDPG al DMP, dando dolicol monofosfato de glucosa (DMPG). El DMP actúa también como aceptor de la N-acetil-glucosamina, a partir de la UDP-N-acetilglucosamina y de manosa a partir de GDP-manosa. La serie de reacciones no termina con este punto, sino que a partir del DMPG se forma otro compuesto que han denominado aceptor glicosilado endógeno. Este compuesto, según un estudio inicial, estaría formado por un hidrato de carbono, conteniendo unas 20 unidades de monosacáridos, unido por un puente fosforado con el dolichol (Leloir, Behrens y Parodi, 1970-1971). Esta sustancia podría tener vinculación con las responsables de los fenómenos de inmunidad celular, con lo cual queda dicho todas las perspectivas que su estudio puede tener.

Creo que expreso el sentir de todos los miembros de nuestra Compañía, al desear al Dr. Leloir, que el éxito lo acompañe en sus nuevas investigaciones, por lo que las mismas pueden significar para crear y ampliar nuevos conocimientos básicos, lo cual, como su propia experiencia lo ha demostrado, representa un beneficio para todos.

DOCTOR LUIS F. LELOIR

Por el Dr. RANWELL CAPUTTO

Semblanza leída con motivo de la entrega del Premio B. H. Houssay del Consejo Nacional de Investigaciones Científicas y Técnicas

La curva de reconocimiento de un hombre de ciencia es muy frecuentemente la curva de un efecto cooperativo donde un mérito refuerza el reconocimiento de otro mérito. En 1946 cuando conocí a Leloir me sorprendía la poca atención que se había prestado a su obra en el país. Ya era fácilmente el más alto exponente de la bioquímica argentina y sin embargo aún no había alcanzado el grado de profesor adjunto de la Universidad de Buenos Aires. Yo tenía la impresión que los méritos de Leloir habían escapado a la atención aún del mismo Dr. Houssay. Por suerte alrededor de 1948 después que fuera invitado a un Symposium sobre metabolismo del fósforo en la Universidad de John Hopkins, el país comenzó a valorarlo. Naturalmente esto ha traido algunas complicaciones para quienes tienen que hablar sobre él. Sin embargo, hasta 1970 hablar de Leloir continuó siendo una tarea relativamente simple. El era un buen bioquímico que nos interesaba principalmente a los bioquímicos. Desde 1970, la situación ha cambiado totalmente. Hoy es muy difícil encontrar un resquicio donde intercalar una palabra nueva sobre su actuación científica. En un número de "Ciencia e Investigación" dedicado a su homenaje, don Venancio Deulofeu, probablemente uno de los amigos que más influencia ha tenido sobre Leloir, describe brevemente su actuación en la A.A.P.C.; Paladini describe el ambiente del Instituto de Investigaciones Bioquímicas en sus comienzos y Carminatti el IIB * moderno. En los Anales de la Sociedad Científica Argentina, Stoppani hace un resumen de la labor científica de Leloir y Olavarría describe lo que él llama el espíritu de IIB. Para completar esta formidable literatura sobre Leloir en IIB, un simposio de carácter internacional organizado por los doctores Pontis y Piras el año pasado comenzó con un relato del mismo Leloir de la obra realizada en IIB y terminó con un estudio de Carl Cori sobre la enorme trascendencia que dicha obra ha tenido en los conceptos sobre la síntesis de materiales biológicos de todo tipo.

Querer agregar algo a todo eso es muy difícil, salvo que alguna vez decidiéramos reunirnos Juan Carlos Fasciolo o Alberto Taquini que lo acompañaron en los estudios de hipertensión experimental, Juan Muñoz que lo acompañó en el estudio del metabolismo de los ácidos grasos, Trucco o Paladini o yo que lo acompañamos en las etapas iniciales del estudio del metabolismo de carbohidratos, Cardini o Cabib y Behrens o Parodi que son sus asociados actuales más cercanos en los estudios sobre biosíntesis de polisacáridos, y todos juntos lo analizáramos no sólo en sus virtudes, sino también en sus defectos, para dejar de él una visión más humana que esta actual, según la cual es trabajador, perseverante, inteligente, generoso y modesto en grado superior. Creo que si a esa descripción, que es, sin duda correcta le agregásemos algunos auténticos defectos, le haríamos a nuestros compatriotas el obsequio de la primera buena descripción de un sabio argentino auténtico que no por sabio dejó de ser humano. Esto por otra parte sería digno de la indudable sinceridad de Leloir. Yo intenté hacerlo por mi cuenta pero me resultó muy difícil. Me faltaban controles de apreciación y me sentía perdido. Por esto, como dije, me limitaré a referir algunos recuerdos personales de mi amistad con él, con algunas apreciaciones que para mí pueden ser útiles para predecir cómo actuaría Leloir en cualquier emergencia. Por cierto no puedo dar garantías de que mi memoria es correcta; todos sabemos que la memoria suele hacernos malas jugadas, pero como recuerdos, son honestos en lo que a mi mejor entender se refiere.

Como dije, conocí a Leloir en el año 1946. No puedo menos que sorprenderme de las extraordinarias similitudes que las incertidum-

* IIB. Instituto de Investigaciones Bioquímicas "Fundación Campomar".

bres políticas de aquél entonces tienen con las de hoy. Sin embargo, por debajo de las apariencias externas la situación ha cambiado mucho y sería peligroso suponer que la evolución de lo que viene va a ser parecida a lo que fue en aquél entonces. Pero Leloir con gran probabilidad va a continuar haciendo lo mismo que hacía en los años que sucedieron a 1946, en que llegaba al laboratorio alrededor de las 9 de la mañana, comenzaba su experimento matinal y sólo cuando éste terminaba total o parcialmente, solía preguntar lo que estaba ocurriendo en el país. Recuerdo que una tarde, temprano, probabemente en el año 1947 ó 48, estábamos en nuestros trabajos habituales cuando corrió la voz de que algo grave estaba ocurriendo en las esferas oficiales y se aconsejó que nos fuéramos a nuestras respectivas casas. Cuando quisimos hacerlo nos encontramos con que el transporte urbano había parado. Leloir era el único del grupo que tenía automóvil entonces y, en un ambiente de intensa preocupación nos distribuyó en lugares adecuados para que llegáramos a nuestras casas. Tuve la suerte de tomar uno de los últimos trenes suburbanos que salían hacia Don Torcuato donde yo vivía. Creo que había 2 ó 3 kilómetros desde la estación a mi casa, que debí caminarlos. En el camino vi un gran despliegue de aparataje militar y oí cuantas versiones se puede uno imaginar, incluyendo la versión de un intento de asesinato al Presidente de la República. A la mañana siguiente cuando llegué al Instituto todavía estaba yo preocupado y quería comentar lo que había visto. Pero en los experimentos de la mañana había aparecido un resultado interesante, como consecuencia de eso se dio un seminario sobre esos resultados y se postergaron los comentarios sobre los incidentes políticos del día anterior. Yo no sabría decir si esta actitud de Leloir es una actitud de egoísmo, o de fundamentalista o simplemente que es una forma de ética del trabajo. Es decir, que no sé si lo hace así porque considera que la ciencia es lo más importante o simplemente que siendo ciencia su trabajo, hacer ciencia es su primera obligación, sea o no importante, pero en cualquier caso, creo que ésta es una característica con la cual se debe contar para predecir su actitud política y tal vez social. Si se dijera, por ejemplo que el país está en una encrucijada y necesita un director para su política científica sería natural en estos momentos que Leloir fuera una de las primeras personas a quien buscáramos para esa función; pero de acuerdo a mi experiencia con él, yo esperaría que su respuesta más probable fuera que él ya dirige su grupo de trabajo en la mejor forma que sabe. En realidad ésta fue su respuesta después de un breve tiempo al frente del Departamento de Química Biológica de la Facultad de Ciencias Exactas, Físicas y Naturales de la Universidad de Buenos Aires y ésta fue también su respuesta hace apenas un par de años cuando un grupo de responsables en la conducción de la ciencia argentina quiso convencerlo de que buscara en forma activa la presidencia del CONICET, que estaba quedando sin la protección de la poderosa personalidad de Houssay, abatido ya por la enfermedad que sus médicos consideraban terminal. La misma característica manifestaba Leloir el año pasado en Bariloche; en un descanso de las reuniones de la Sociedad Argentina de Investigación Bioquímica se quejaba de que casi todos los días le llevaban un proyecto que significaba el gasto de una enorme cantidad de dinero y le pedían su apoyo para que fuera llevado adelante, y qué podía ayudar él!, si generalmente no le daban tiempo para enterarse del contenido de uno cuando ya le traían el siguiente. Cuando alguien le sugirió que debía dedicarse a estudiar esos proyectos más que a trabajar en el laboratorio hizo uno de esos gestos, muy suyos, que yo interpreto que significan que no hay que perder tiempo en discutir sonseras.

Cuento estas actitudes de Leloir, tal como yo las he percibido, no como elogio o crítica a la actitud en sí, sino simplemente como descripción. Creo, sin embargo, que hay que señalarlo por el tremendo valor que tienen como elemento de producción y por la gran valentía personal que significa mantenerse dentro de ellas, contra toda la masa de supuestos amigos que tratan de sacarlo de las mismas. Ha sido para mí un grato espectáculo verlo muchas veces en medio de grupos que querían sacarlo de su actitud y él, siempre el de apariencia más débil, con una mente aparentemente abierta a todas las sugestiones, terminar siempre sin una definición lo que significaba a la larga su definición.

Incluido en la acción de Leloir como bioquímico está su labor docente a nivel superior y no hay dudas que también ha hecho esto con eficacia. Basta fijarse en el número de profesores de bioquímica que fueron sus colaboradores para quedar de acuerdo con esta apreciación. Su forma de enseñar es muy particular, no sujeta a hábitos y tradiciones, y sólo después de bastante tiempo nos dimos

cuenta que estaba enseñando. Pero no quiero referirme a eso ahora, sino simplemente contar una de las anécdotas de IIB que además de encontrarla divertida me parece que muestra uno de los aspectos más agradables del espíritu pionero con que se inició este instituto. Me refiero al afán de formar el "Faraday Argentino". Faraday, según la tradición, se inició en el trabajo de laboratorio como un joven peón de limpieza en el laboratorio de Davy. La broma, o lo que fuera, del "Faraday argentino" había flotado en el ambiente de IIB por un tiempo cuando Leloir anunció que había tomado un joven de 17, 18 años para efectuar la limpieza del laboratorio y anunció su esperanza de haber encontrado un futuro genio de la ciencia argentina. Juan Carlos, así se llamaba el motivo de la esperanza, era un joven agradable de aspecto y maneras, despierto y probablemente más capaz que el término medio de los jóvenes de su edad. Comenzó muy bien y todos estábamos felices que la predicción podría cumplirse. Poco tiempo después sin embargo, los problemas de la edad de J. C. se hicieron evidentes y su trabajo declinó mucho; los miembros del Instituto empezaron a reclamar que se lo reemplazara por algo que significara una esperanza menor pero una mayor eficiencia en el trabajo del momento. Leloir se resistió un tiempo pero finalmente debió ceder y anunció que despediría a Juan Carlos. Lo llamó y estuvieron conversando a solas por alrededor de media hora. Conversación tan prolongada era desusada en Leloir y nos llamó la atención, pero al terminar Leloir volvió a su laconismo habitual. Su versión de lo conversado fue que le había dicho a Juan Carlos que buscara otro trabajo y se fuera. La versión de Juan Carlos fue más exhuberante: "¡Fantástico!, el Dr. Leloir está muy contento conmigo y me dijo que estudiara Química; me contó algo de un inglés famoso que no me acuerdo como se llamaba pero que había empezado igual que yo y después ganó toda la plata que quiso. Doctor, me presta un libro de Química...". No sé a quien le tocó eventualmente despedir a Juan Carlos, pero Leloir tuvo que cargar sobre sus hombros el cuento del Faraday argentino durante muchos meses.

Yo diría que todo el grupo inicial de IIB tenía una gran dedicación, integral más allá de lo usual, al trabajo de investigación bioquímica. Esto, naturalmente, no ayudaba a hacer un grupo particularmente culto en el sentido general de esta palabra, éramos, más bien, algo de lo que se puede llamar técnicos semi-bárbaros y esto nos enajenaba del medio ambiente y nos traía algunos reproches de nuestras respectivas señoras. Aquí tal vez sea pertinente un recuerdo sobre la señora de Leloir. Como todas las otras apreciaciones que registro aquí, son mías y pueden no coincidir con las de los otros. Doña Amelia venía a buscar a su marido al laboratorio yo diría que casi todas las tardes. Recuerdo que gozábamos a veces con las gracias de los primeros pasos de Amelita, la chiquita del matrimonio que debe ser sólo 1 ó 2 años menor que IIB. Yo me sentía un poco corto frente a doña Amelia no sólo por mí sino también por todo el grupo, por esta condición de técnicos cerrados a otras manifestaciones del espíritu que no fueran conocimientos químicos, frente a esta señora que era culta, que leía libros, apreciaba arte, teatro, danzas. Yo creo que detrás de la gracia de su sonrisa permanente debía sufrir al vernos tan irremediablemente perdidos a todo lo que fuera cultura general. Una vez me regaló un libro: "La Hora 25". Me aconsejó que lo leyera y lo hice. La verdad es que me interesó mucho, pero lo importante a decir en este momento, y ojalá no esté diciendo una herejía, es que creo que desde entonces llevo por lo menos un libro de ventaja sobre su esposo en lectura de temas de interés general, aunque no tengo dudas que él me lleva la ventaja de la lectura de muchos libros de química.

Pero esto no significa que no se transparenten en Leloir algunos elementos de dudas con respecto a su labor. Tan unido y coherente como aparece en su trabajo es posible que haya algo por lo cual creo que se hubiera diversificado al primer contacto con un éxito que lo hubiera empujado en ese sentido. En algunos círculos he oído decir alguna vez que para el hombre de ciencia el finalismo es una amante, con quien le agrada estar, pero con quien no quiere ser visto. Yo creo que para Leloir la aplicación es algo de esa naturaleza. La aplicación de la ciencia es algo que no figura en sus trabajos pero es probable que esto le ha interesado a lo largo de una buena parte de su carrera. Recuerdo que durante los primeros tiempos de IIB él incorporó al laboratorio a una joven bioquímica para que estudiara un método de purificación de la heparina. Nunca supe cuál sería el eventual destino de los resultados del trabajo de esa señorita pero se aludía mucho, mitad en broma, mitad en serio, en que el futuro económico de IIB dependía de la obtención de una

buena heparina. Tiempo después Leloir apareció con la sugestión de que buscáramos un substituto del glicerol, según creo, en la elaboración de pastas dentífricas. En la única aparición de Leloir que yo conozco como editorialista de "Ciencias e Investigación", sugiere que nuestro país debiera tomar el ejemplo de una zona de pequeña industria alrededor de Boston donde se producen, si recuerdo bien, materiales para uso en la investigación científica. Pero el recuerdo de él que más me impresionaba en este aspecto de su personalidad, no sólo como ejemplo de genuina modestia sino también como la confesión nostálgica de un primer cariño, es una conversación que mantuvimos en Córdoba. Leloir nos estaba haciendo el favor excepcional de ir allá como jurado en un concurso. Probablemente fue la única vez en su vida que ha hecho un viaje para participar en una actividad que es más burocrática que académicamente útil. En el momento que voy a referir estábamos sentados en un antiguo baño transformado en el lugar donde se prepara el café, se destila el agua que usamos en el laboratorio y sirve de depósito de material de limpieza. El lugar es un tanto sucio y desagradable y yo me sentía algo avergonzado de tenerlo bebiendo café allí. Leloir, sin embargo, parece sentirse cómodo en esos lugares en los que el trabajo se junta con la pobreza y es en ese ambiente donde se puede extraer lo más genuino de él. Hablábamos de ese estribillo de "ciencia al servicio de la comunidad" que parecía que lo estaba convenciendo. Yo siempre he temido que el tal estribillo sea más dañino que servicial y por lo tanto me interesaba saber cómo lo aplicaría él si alguna vez decidiera hacerlo. Su respuesta fue algo así: ..."y, qué sé yo, tome mi caso por ejemplo, tantos años trabajando en estas cosas y nunca he encontrado algo que ayude al "país". La respuesta me tomó por completo de sorpresa y casi no podía creer lo que oía, pero continué con el tema y le pregunté en qué forma creía él que podía hacer algo que ayudara al país; la respuesta volvió enseguida: "...bueno, debe haber infinidad de cosas que hacer: por ejemplo, pudimos haber estudiado un método de esterilización de latas de carne mejor que los que tenemos ahora; seguro eso ayudaría al País". No hay dudas, yo me siento tan seguro como él de que eso ayudaría al país y estoy dispuesto a atribuirle todos los méritos que se gane el investigador que encuentre ese método, pero dudo mucho que al correr del tiempo pudiera competir con lo que Leloir había hecho hasta aquel momento en beneficio del país. Cuando manteníamos esta conversacion ya se podía aplicar a Leloir las palabras que dijo Carl Cori en Bariloche el año pasado: ..."comenzando en 1941 con la identificación del UDPG como un cofactor necesario en la conversión de glucosa a galactosa ha tenido lugar uno de los más dramáticos desarrollos de la química moderna". Por lo que al país respecta yo diría que en ese mismo período tuvo lugar entre nosotros el mayor desarrollo de los conocimientos químicos, que si bien fueron impulsados en gran parte por la acción docente directa de Deulofeu y su grupo, lo fueron también porque los descubrimientos en I I B bajo la dirección de Leloir despertaron la imaginación de una parte de la juventud argentina y provocaron un tipo de entusiasmo desconocido hasta entonces. Puede quedar tranquilo el Dr. Leloir: es poco probable que un buen método de esterilización, por útil que sea, pueda provocar una reacción más útil que la que provocó su obra.

En la entrega del Premio Houssay a Leloir no podemos evitar hacer referencias a los resultados de valores similares que obtuvieron estos dos hombres de estilos tan distintos. Es evidente que desde los tiempos de la formación de Houssay a los de la formación de Leloir los conceptos de solemnidad y de autoridad habían perdido mucho prestigio. Cuando se recuerdan las apariencias externas de sus respectivas maneras de actuar uno piensa que el conflicto entre aquellas dos generaciones era tan grande como el que existe ahora entre nosotros y nuestros jóvenes. Sin embago, los conceptos fundamentales de estimación al esfuezo, al tabajo y a lo que entonces se llamaba progreso continuaban intactos. Hoy los problemas sobre solemnidad y autoridad están resueltos: la desvalorización será permanente por muchos años. Pero los jóvenes están ahora en dudas sobre si hay que entrar a un mundo de felicidad sin esfuezo o hay que persistir en la premisa de que el trabajo produce las únicas satisfacciones profundas. En medio de un ambiente de descontento y de un sentido de frustraciones exagerado, la labor continuada de Houssay primero y de Leloir después a lo largo de 50 años, nos han dado las mayores satisfacciones, y las más limpias de problemas, que ha experimentado este país desde 1947 hasta la fecha. En el caso de Leloir nosotros hemos visto que los jóvenes de la

Universidad de La Plata en 1970 y los de Corrientes en 1972 lo señalaban con orgullo y satisfacción en cualquier lugar que lo descubrieran. Esto es un rayo de esperanzas en una situación que por tantos motivos nos tiene azorados a quienes mantenemos nuestra fe en los conceptos tradicionales. La obra de Leloir quedará sin duda como un capítulo indeleble en el relato de los conocimientos de biología y eso quedará como su contribución de interés universal. En nuestro país, su obra y su ejemplo han excitado la imaginación de muchos jóvenes que quieren igualarlo y aún superarlo. La masa de jóvenes que están distribuidos en docenas de laboratorios en todo el país constituyen, con Houssay y Leloir, la tríada a la que está dedicado el homenaje de esta tarde.

Luis F. Leloir en el balcón de su residencia con su esposa, Amelia Zuberbuhler, y su hija Amelita.

VEINTE AÑOS DE INVESTIGACION SOBRE LA BIOSINTESIS DE POLISACARIDOS

LUIS FEDERICO LELOIR

Nuestro trabajo en la biosíntesis de oligosacáridos y polisacáridos comienza en 1946, no por una elección deliberada del tema sino en forma casual. Debido al fenomenal progreso de la bioquímica, nuestros primeros experimentos parecen pertenecer a la era paleolítica; pero, afortunadamente, existen también algunos muy recientes e interesantes progresos en este campo.

Al volver de Cambridge, en 1936, realicé algunos trabajos con J. M. Muñoz sobre la oxidación de ácidos grasos en el hígado. Nos fue posible preparar un sistema libre de células que era activo en presencia de cofactores necesarios. Este fue un resultado novedoso ya que entonces se creía que la oxidación de ácidos grasos requería la integridad celular.

Supongo que a la nueva generación de bioquímicos le es difícil comprender muchas de las cosas en las que creíamos nosotros en ese tiempo.

Después de esto, hice una incursión en el campo de la hipertensión renal con E. Braun Menéndez, J. C. Fasciolo y A. C. Taquini. Este trabajo fue llevado a cabo rápidamente y con cierto éxito. Luego trabajé en el Laboratorio de Carl Cori, en St. Louis, y con D. E. Green, en la Universidad de Columbia.

A mi regreso a Buenos Aires en 1945, empecé a trabajar con R. Caputto y R. Trucco. El doctor Caputto había hecho algunas investigaciones en glándula mamaria y tenía la idea de que el glucógeno se transformaba en lactosa. En aquel tiempo teníamos que usar osazonas para la identificación de los azúcares y muy pronto llegamos a un punto muerto. Mirando atrás, creo que lo que estábamos observando era la degradación del glucógeno por la amilasa.

Conferencia pronunciada por el Dr. Leloir en Estocolmo al recibir el Premio Nobel de Química, 1970.

Decidimos entonces estudiar la degradación de la lactosa por la levadura, *Saccharomyces fragilis*, con la idea de que esto nos daría una información sobre el mecanismo de su síntesis. Finalmente se obtuvo esa información, pero sólo a través de un camino largo y tortuoso.

Primero estudiamos la lactasa, después la fosforilación de la galactosa [1] y la trasformación de la galactosa fosfato en glucosa fosfato. Lo que medíamos era el incremento de poder reductor resultante de la siguiente secuencia de reacciones:

Galactosa 1-fosfato → glucosa 1-fosfato → glucosa 6-fosfato (1)

Pronto encontramos que para que la reacción tuviese lugar se requería la presencia de un cofactor termoestable y nos propusimos aislarlo, en colaboración con C. E. Cardini y A. C. Paladini.

En ese entonces las cosas no eran muy fáciles, debido a que no disponíamos de los métodos de que disponemos hoy y además, porque trabajábamos en condiciones bastante precarias.

Los resultados de nuestros experimentos fueron muy poco claros porque no nos dimos cuenta de que eran dos los cofactores involucrados. Finalmente descubrimos lo que pasaba y nos concentramos en la purificación del cofactor de la segunda reacción, es decir, la reacción catalizada por la fosfoglucomutasa.

Enviamos una carta a los editores de los *Archives of Biochemistry* [2] describiendo al nuevo cofactor y mencionando que Kendall y Strickland [3] habían descripto previamente una activación por fructosa 1,6-difosfato pero que nuestro cofactor era diferente. Después de enviar el manuscrito ensayamos la fructosa 1,6-difosfato y obtuvimos una fuerte activación. Más aún, nuestras preparaciones purifi-

Luis F. Leloir recibe el Premio Nobel de Química 1970, que le entrega el Rey Gustavo de Suecia en una solemne ceremonia en la Academia de Ciencias de Estocolmo.

cadas estaban fuertemente contaminadas con fructosa 1,6-difosfato. Ya habíamos decidido solicitar se nos devolviera la carta, cuando como consecuencia de tanto preocuparnos surgió la idea de que el activador debía ser glucosa 1,6-difosfato. En vista de que este último compuesto tiene bloqueado el grupo reductor, pensamos que debería ser estable en medio alcalino y, curiosamente, todo salió como esperábamos. Si no hubiera sido por ese error, podríamos estar aun hablando del efecto alostérico de la fructosa 1,6-difosfato sobre la fosfoglucomutasa.

Cuando acabamos de trabajar con la glucosa 1,6-difosfato continuamos con el otro cofactor. Se encontró que los concentrados absorbían la luz a 260 nanometros y tenían un espectro similar al de la adenosina, pero con ciertas diferencias. En ese entonces los únicos nucleótidos solubles presentes en el tejido que se conocían eran los ácidos adenílicos y el inosínico. Fue muy emocionante el día en que Caputto llegó temprano con un ejemplar del *Journal of Biological Chemistry*, el cual mostraba el espectro de la uridina. Este era idéntico al de nuestro cofactor. Después de medir el contenido glucosa y fosfato, y realizar una curva de titulación, se propuso la estructura que se muestra en la Fig. 1 [4]. El primer nucleótido-azúcar se llamó uridina difosfato glucosa: UDPG. Su estructura fue confirmada por síntesis alrededor de 5 años más tarde por Todd y colaboradores, en Cambridge. El mecanismo por el cual UDPG-glucosa actúa de co-factor en la transformación de galactosa-1-fosfato en glucosa-1-fosfato se aclaró cuando se encontró que incubando con extractos de levadura parte de la UDP-glucosa se trasformaba en UDP-galactosa [5]. Después de esto, escribimos las ecuaciones como sigue:

$$\text{galactosa 1-foofato} + \text{UDP-glucosa} \rightleftharpoons \text{glucosa 1-fosfato} + \text{UDP-galactosa} \qquad (2)$$

$$\text{UDP-galactosa} \rightleftharpoons \text{UDP-glucosa} \qquad (3)$$

$$\text{Suma: galactosa 1-foofato} \rightleftharpoons \text{glucosa 1-fosfato} \qquad (4)$$

Llamábamos a todo el sistema waldenasa, pero Kalckar [6] sugirió llamar uridilil transferasa y 4-epimerasa a las enzimas correspondientes a las ecuaciones (2) y (3), respectivamente.

Cuando encontramos que las levaduras no adaptadas a la galactosa contenían un alto nivel de UDP-glucosa, dedujimos que la UDP-glucosa debería tener otra función, además de la de cofactor del metabolismo de la galactosa. No sé si el razonamiento era correcto, pero los hechos nos dieron la razón. Estuvimos durante varios años preguntándonos. ¿Para qué sirve el UDPG?, y la pregunta se convirtió en una broma, en el laboratorio.

Como teníamos un método para medir la UDP-glucosa basado en su acción sobre la velocidad de la reacción galactosa 1-fosfato → glucosa 6-fosfato, empezamos a medir la desaparición de UDP-glucosa en diferentes extractos y bajo diferentes condiciones. Con extractos de levadura se observó que la adición de glucosa 6-fosfato incrementaba la desaparición de UDP-glucosa lo que finalmente se demostró que era debido a la formación de trehalosa fosfato, una sustancia que había sido aislada de la levadura muchos años antes por Robison y Morgan [7].

La reacción es la siguiente:

UDP-glucosa + glucosa 6-fosfato →
trehalosa-fosfato + UDP (5)

Este trabajo, llevado a cabo con Cabib [8], describía el primer caso en que se observó que la UDP-glucosa actuaba como dador de glucosa. Esto había sido sugerido por Buchanan *et al* [9] y por Kalckar [10].

Una vez descubierta una reacción de transferencia, pronto pudimos detectar otra usando extracto de germen de trigo. En realidad, lo que encontramos fueron dos enzimas, una que llevaba a la formación de sacarosa [11], y otra que daba sacarosa-fosfato [12] como sigue:

DP-glucosa + fructuosa → sacarosa + UDP (6)

UDP-glucosa + frictosa 6-fosfato →
sacarosa fosfato + UDP (7)

Este hallazgo realizado con Cardini y Chiriboga fue también interesante dado que aclaraba el mecanismo de la síntesis de sacarosa en vegetales.

Otro resultado novedoso de aquel período fue el aislamiento del UDP-acetilglucosamina [13]. Esta sustancia se detectó primero como una impureza de los concentrados de UDP-glucosa y la solíamos llamar UDP-X hasta que pudimos identificar el resto de azúcar como *N*-acetilglucosamina. Se sabe ahora que interviene en la biosíntesis de la pared celular de las bacterias y de las mucoproteínas.

Otros miembros de la familia de los nucleótidos-azúcares fueron aislados en nuestro laboratorio. En 1954 con Cabib [14] se encontró GDP-manosa en extractos de levadura, y más tarde Pontis [15] detectó UDP-acetilgalactosamina en el hígado. Se sabe ahora que estas sustancias intervienen en la biosíntesis del manano [16] y de algunas glucoproteínas.

Otros laboratorios realizaron importantes contribuciones. La identificación de UDP-ácido glucurónico como dador en la formación de glucurónidos [17] fue el primer ejemplo de una reacción de transferencia a partir de un nucleótido-azúcar.

Otro compuesto importante fue descubierto por Park y Johnson [18] casi al mismo tiempo que aislamos el UDP-glucosa. Ellos observaron que en *Staphylococcus* tratado con peni-

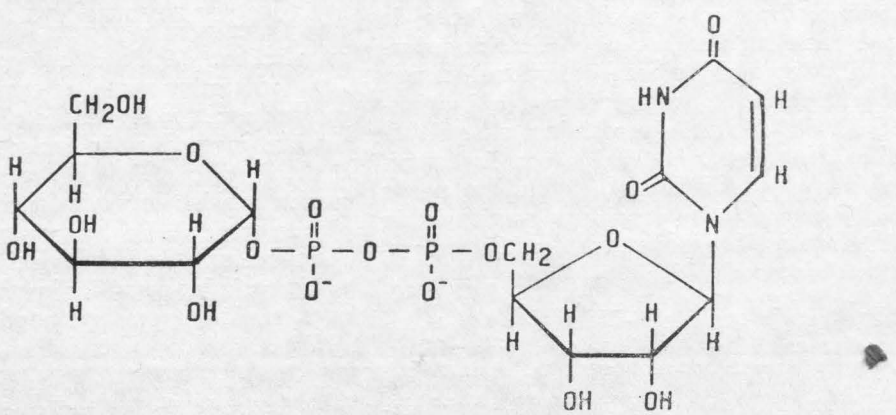


FIG. 1. — Uridina-difosfato-glucosa (UDPG).

cilina se acumula un compuesto que contiene uridina. Esta sustancia resultó difícil de identificar debido a que su resto de azúcar no se conocía en ese entonces. Este compuesto se comportaba como una hexosamina desconocida. Strange y Dark [19] fueron los primeros en obtener una preparación cristalina. Se sabe ahora que el resto de azúcar presente es la acetilglucosamina combinada por una unión eter con ácido láctico. Esta sustancia se llamó ácido murámico. El aislamiento del UDP-ácido murámico fue el punto de partida del trabajo tan interesante de Park y Strominger sobre síntesis de la pared celular de las bacterias.

El número de los nucleótido-azúcares conocidos aumentó progresivamente en el curso de los años, y, en la recopilación realizada en 1963 eran ya más de 48 [20]. Además, se estudiaron muchas enzimas que intervienen en reacciones de interconversión. El grupo de Herman Kalckar encontró que se requería NAD en la reacción de la UDP-glucosa 4 epimerasa y se cree que el resto de glucosa del UDP-glucosa se oxida a un 4-ceto intermediario que puede reducirse tanto a glucosa como a galactosa.

Algunas otras transformaciones más complicadas han sido estudiadas cuidadosamente; por ejemplo, la transformación de GDP-manosa a GDP-fucosa, que requiere una reducción en C-6 e inversiones en C-3 y C-5 [21]. Un caso similar es la formación de TDP-ramnosa partiendo de TDP-glucosa, en la cual los grupos OH en C-3, C-5 y C-6 se invierten y ocurre una reducción en C-6 [22].

Polisacáridos

Se han detectado muchas reacciones de transferencia partiendo de nucleótido-azúcares. Así, Glaser y Brown [23] encontraron una transferencia de *N*-acetilglucosamina partiendo del UDP-*N*-acetilglucosamina a quitina catalizada por extractos de hongos. La formación de un β-1,3 glucano (callosa) a partir de UDP-glucosa y la de xilano partiendo de UDP-xilosa se obtuvo por incubación con extractos de vegetales [24].

Una transferencia de UDP-glucosa a celulosa fue también descripta por Glaser [25] en *Acetobacter xilinum*, una bacteria formadora de celulosa. Más tarde se descubrió que el dador para la formación de celulosa en las plantas es la GDP-glucosa.

En nuestro laboratorio pudimos observar la formación de glucógeno por enzimas provenientes de hígado y músculo partiendo de UDP-glucosa:

$$\text{UDP-glucosa} + G_n \rightarrow \text{UDP} + G_{n+1} \qquad (8)$$

En esta ecuación G_n representa una molécula de glucógeno y G_{n+1} la misma molécula después de la adición de un residuo glucosilo en unión α-1,4. La búsqueda de esta enzima, glucógeno sintetasa o transferasa, fue estimulada por la lectura de un libro escrito por Herman Niemeyer [28], y su descubrimiento fue de interés, puesto que hasta entonces se creía que la síntesis del glucógeno se producía por reversión de la reacción de fosforilasa (ec. 9):

$$(\text{glucosa})_{n+1} + \text{fosfato inorgánico} \rightleftharpoons (\text{glucosa})_n + \text{glucosa 1-fosfato} \qquad (9)$$

Se creía entonces que esta enzima actuaba tanto en la síntesis como en la degradación del glucógeno. Otro descubrimiento de considerable interés fue que la glucosa 6-fosfato actúa como un activador del glucógeno sintetasa.

Muchos años antes los Cori habían encontrado que la fosforilasa del músculo existía en dos formas que se diferenciaban por su requerimiento de ácido adenílico. En forma similar, J. Larner y C. Villar-Palasi describieron dos formas interconvertibles de glucógeno sintetasa, una activa *per se* y otra que requiere glucosa-6-fosfato. Desde entonces, se han realizado muchos estudios sobre la regulación del metabolismo del glucógeno.

Tanto la fosforilasa como el glucógeno sintetasa son reguladas por la concentración de metabolitos (ácido adenílico y glucosa-6-fosfato, respectivamente, y también otros compuestos como, por ejemplo el ATP) y por conversión reversible de formas activas a inactivas. Los cambios posteriores se producen por la acción de varias enzimas que interaccionan entre sí. La imagen que tenemos sobre el mecanismo de la regulación del glucógeno es demasiado complicada para mostrarla en esta ocasión (para un estudio detallado ver (29)).

Casi todas las investigaciones realizadas en la biosíntesis de los polisacáridos han consistido solamente en la medición de la transferencia de cantidades muy pequeñas de azú-

cares radioactivos. Sin embargo, los estudios deberían ampliarse hasta poder obtener *in vitro* polisacáridos idénticos a aquellos que producen las células. Algunos trabajos de este tipo han sido realizados con el glucógeno. Se puede obtener glucógeno incubando glucosa 1-fosfato con fosforilasa o a partir de UDP-glucosa con glucógeno sintetasa (en ambos casos en presencia de la enzima ramificante). Los productos así obtenidos son de alto peso molecular, pero diferentes a juzgar por sus características de degradación por ácido o alcalí. El producto formado por UDP-glucosa y glucógeno sintetasa es idéntico a aquel que se aisla del hígado [30].

Una extensión lógica de nuestros estudios sobre el glucógeno fue investigar la formación del almidón en las plantas. Se encontraron enzimas que catalizan la transferencia al almidón de glucosa radiactiva del UDP-glucosa marcada en su resto de glucosa [31]. Estudios sobre la especificidad de la enzima usando nucleótidos sintéticos mostraron que el ADP-glucosa es utilizado diez veces más rápido que el UDP-glucosa [32]. Esto llevó a la búsqueda de ADP-glucosa en fuentes naturales y se logró aislarlo del maíz [33]. Una enzima capaz de sintetizar el ADP-glucosa fue encontrada por Espada [34].

A partir de entonces se han realizado muchas investigaciones sobre el tema, especialmente por C. E. Cardini, Rosalía Frydman, Jack Preiss, T. Akazawa y otros.

En la *Euglena* el polisacárido de reserva es un glucano con uniones β1-3, usualmente llamado paramilon. Su síntesis fue estudiada por Goldemberg y Marechal [35] quienes encontraron que se forma a partir UDP-glucosa.

Con el andar del tiempo se describieron muchas otras reacciones de transferencia por lo que su búsqueda se convirtió en una tarea algo monótona.

Intermediarios lipídicos

Los datos acumulados indicarían que la mayoría de los di-, oligo-, y polisacáridos que en una sorprendente variedad existen en la naturaleza, se sintetizan a partir de nucleótido azúcares. Sin embargo, al menos algunos casos, parecería que la transferencia no es directa sino que es mediada por intermediarios de naturaleza lipídica. Este es uno de los más importantes descubrimientos de los últimos años y está vinculado a la labor de varios grupos (Osborn, Horecker, Strominger, Robbins, Lenartz y otros). El primer intermediario lipídico fue detectado en bacterias [36] y se muestra en la figura 2.

La estructura del compuesto fue resuelta mediante el análisis por espectroscopía de masa de muy pequeñas cantidades. El compuesto, undecaprenol pirofosfato, contiene 11 residuos isoprenos uno de los cuales lleva un grupo OH unido a un pirofosfato que a su vez se une a los restos de azúcar.

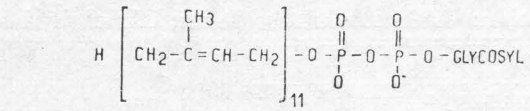


FIG. 2.— Antígeno intermediario lipídico.

El papel del lípido intermediario en la formación de lipopolisacárido de *Salmonella* puede resumirse en las siguientes ecuaciones (donde LP representa el intermediario lipídico monofosforilado):

$$\text{LP} + \text{UDP-galactosa} \rightarrow \text{LPP-galactosa} + \text{UMP} \quad (10)$$

$$\text{LPP-galactosa} + \text{TDP-ramnosa} \rightarrow \text{LPP-galactosa-ramnosa} + \text{UDP} \quad (11)$$

$$\text{LPP-galactosa-ramnosa} + \text{GDP-manosa} \rightarrow \text{LPP-galactosa-ramnosa-manosa} + \text{GDP} \quad (12)$$

$$n\ \text{LPP-galactosa-ramnosa-manosa} \rightarrow \text{LPP (galactosa-ramnosa-manosa)}_n + (n-1)\ \text{LPP} \quad (13)$$

$$\text{LPP (galactosa-ramnosa-manosa)}_n + \text{core} \rightarrow \text{(galactosa-ramnosa-manosa)}_n\ \text{core} + \text{LPP} \quad (14)$$

$$\text{LPP} \rightarrow \text{LP} + \text{P} \quad (15)$$

El primr paso (ec. 10) consiste en una transferencia de galactosa 1-fosfato formándose pirosfato lipídico y UMP. Después se agregan la ramnosa y manosa por transferencia del respectivo nucleótido azúcar. La transferencia final se hace por unidades de trisacáridos y se forman cadenas largas (n = alrededor de 60) de unidades repetidas de galactosa-ramnosamanosa unidas al intermediario. En el siguiente paso (ec. 14) ellas se transfieren al núcleo del lipopolisacárido.

El undecaprenol pirofosfato juega un papel similar en la formación de la pared celular bacterina del estafilococo. El material de la pared, la mureina, está formada por unidades alternantes de acetilglucosamina y de ácido murámico. Esas cadenas están entrecruzadas por peptidos unidos a los residuos de ácido murámico.

El mecanismo por el cual la pared celular se une fue explicado principalmente por el trabajo realizado por el grupo de Strominger [37] y se puede escribir como sigue: (M = *N*-acetil ácido murámico unido al siguiente peptido: L-ala D-gluL-lys D-ala; *N*-Ac significa N-acetilglucosamina) :

$$\text{UDPM} + \text{LP} \rightarrow \text{LPPM} + \text{UMP} \qquad (16)$$

$$\text{UDP}N\text{-Ac} + \text{LPPM} \rightarrow \text{LPPM}N\text{-Ac} + \text{UDP} \qquad (17)$$

$$\text{tRNA gly} + \text{LPPM}N\text{-Ac} \rightarrow \text{tRNA} + \text{LPPM}N\text{-Ac gly} \qquad (18)$$

$$\text{LPPM}N\text{-Ac gly} + \text{aceptor} \rightarrow (\text{M-}N\text{-Ac gly})\text{-aceptor} + \text{LPP} \qquad (19)$$

El primer paso (Ec. 16) es una transferencia de muramil peptido fosfato de su correspondiente nucleotido uridina (uno de los compuestos aislados por Park) a undecaprenol monofosfato. A continuación, (Ec. 17) la *N* acetilglucosamina es transferida a partir de UDP-N-acetilglucosamina. Después (Ec. 18) se agrega un aminoácido más (de un ácido ribonucleico de transferencia y entonces se agrega el total de peptido disacárido a una parte de la pared celular en crecimiento (que figura como aceptor en la ec. 19). Después de esto se establecen los entrecruzamientos entre las cadenas de peptidos y la pared celular se completa.

También debe ser mencionado otro trabajo que tiene que ver con los intermediarios lipídicos. Me refiero a la formación de manano por *Micrococcus lysodeikticus* [38]. Las reacciones son:

$$\text{GDP-manosa} + \text{undecaprenol-P} \rightarrow \text{GDP} + \text{undcaprenol-P-manosa} \qquad (20)$$

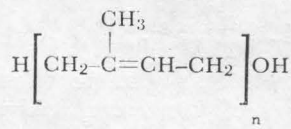
$$\text{undecaprenol-P-manosa} + \text{aceptor} \rightarrow \text{manosa-aceptor} + \text{undecaprenol-P} \qquad (21)$$

La diferencia entre los casos ya mencionados es que en la primera reacción (Ec. 20) el azúcar es transferida sin el fosfato de manera que no se forma pirofosfato sino un monofosfato.

Mientras estas investigaciones se realizaban, Dankert, que había estado trabajando con el grupo de Robbins, volvió a Buenos Aires y nos trasmitió su entusiasmo por los polipreno-les.

Un poliprenol intermediario en tejidos animales

Un grupo formado por Morton, Hemming y otros, en la Universidad de Liverpool ha estudiado cuidadosamente los diferentes poliprenoles que se hallan en la naturaleza. Su fórmula general es la siguiente:



Una cantidad de diferentes compuestos de este tipo fueron detectados, que diferían en el número *n* de residuos de isoprenos, en la cantidad de doble ligaduras *cis* o *trans* y también en que algunas de las doble ligaduras pueden estar saturadas.

El compuesto aislado de tejidos animales fue llamado dolicol. En esta sustancia el número de unidades de isopreno es de alrededor de 20 (puede variar de 16 a 23) y dos de las dobles ligaduras son *trans*. Además, la doble ligadura más cercana al grupo alcohol está saturada. Muchos otros compuestos fueron aislados en otros sistemas [39]. Con N. Behrens [40] hemos estudiado un proceso que ocurre en el hígado y en el cual resultó involucrado un fosfato de dolicol. Las reacciones se pueden escribir como sigue:

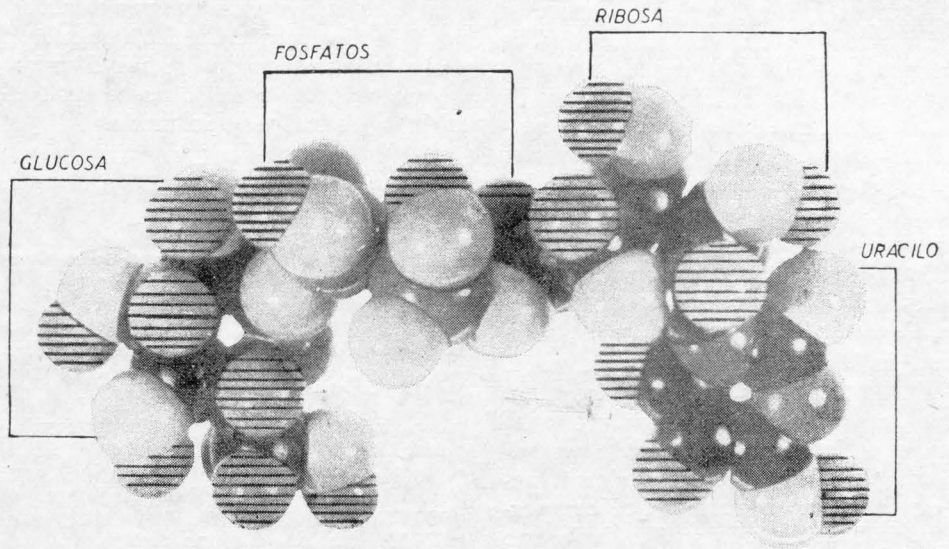
$$\text{UDP-glucosa} + \text{DMP} \rightarrow \text{DMP-glucosa} + \text{UDP} \qquad (22)$$

$$\text{DMP-glucosa} + \text{E} \rightarrow \text{glucosa-E} + \text{DMP} \qquad (23)$$

$$\text{Glucosa-E} \rightarrow \text{glucosa} + \text{E} \qquad (24)$$

En estas ecuaciones DMP significa dolicol monofosfato y E un aceptor endógeno.

Los estudios fueron realizados incubando microsomas de hígado con UDP-glucosa radiactiva. Se encontró que se formaba un producto soluble en solventes orgánicos. Trabajos posteriores mostraron que la reacción de la ecuación 22 podía ser realizada de modo tal como para medir el aceptor lipídico (DMP en la ec. 22). Esto permitió el desarrollo de un proceso de purificación. Los concentrados



Modelo atómico de la molécula del Uridina Difosfato Glucosa

que se obtenían daban un espectro infrarrojo similar al de los poliprenoles. El compuesto tenía un carácter ácidico y era relativamente estable al ácido y al álcali. Difiere del undecaprenol fosfato en que este último es ácido labil. Se dedujo que esta diferencia podría deberse al hecho que en el undecaprenol existe una doble ligadura cercana al fosfato y que no está presente en el dolicol. Teniendo esto presente ensayamos otros enfoques para la identificación de nuestro aceptor lipídico. Se aisló dolicol de hígado [41] se lo fosforiló químicamente y se probó su actividad como aceptor lipídico. El compuesto sintético resultó ser idéntico en todas sus propiedades al obtenido de fuentes naturales. Por esta razón nos referimos a él como dolicol monofosfato.

En cuanto al compuesto glucosilado (DMP-glucosa) se encontró que era muy lábil en medios ácidos y que se descomponía en medios alcalinos, dando 1,6-anhidro-glucosano. La reacción 23 se pudo estudiar independientemente de la primera usando DMP-glucosa preparada en una etapa previa. Se determinaron las condiciones óptimas para actividad. Este paso (Ec. 23) no requiere ningún catión, contrariamente a la reacción que se muestra en Ec. 22, en la cual es necesario el ión Mg^{24}.

El producto formado a partir de DMP-glucosa y que se indica como glucosa-E en la Ec. 23, se creyó al principio que era una proteína glucosilada, pero nuestro trabajo en este aspecto recién ha comenzado. Hay pocas proteínas que contienen glucosa. Una de ellas es el colágeno que contiene residuos de glucosil, galactosil hidroxi-lisina. Sin embargo, el compuesto formado con microsomas de hígado parece diferenciarse claramente del colágeno. La última reacción (Ec. 24) no ha sido estudiada en detalle y podría ser producida por alguna de las glucosidasas que se sabe están presentes en el hígado.

Se estudió la posibilidad de que la glucosilación de ceramida, que es el primer paso en la formación de gangliosides, podría estar mediado por DMP-glucosa y los resultados, aunque no enteramente conclusivos, indican que no está involucrada la DMP-glucosa.

Se probaron otros nucleótido-azúcares y se encontró que UDP-*N*-acetilglucosamina y GDP-manosa pueden servir como donores en la formación del correspondiente DMP-azúcares. Otros compuestos como UDP-*N*-acetil-galactosamina y UDP-galactosa dieron resultados negativos [42].

El estudio de los intermediarios lipídicos está resultando muy interesante. La variedad de los poliprenoles es muy grande, pues pueden diferir en la longitud de la cadena, número de doble ligaduras *cis* o *trans* en su grado de saturación. Además, pueden tener

uno o dos grupos fosfatos y llevar diferentes azúcares. La variedad de los azúcares poliprenol fosfatos puede ser tan grande como la de los nucleótido-azúcares. Ha sido sugerido que su rol podría ser el proveer de una porción lipofílica a los azúcares para permitirles su pasaje a través de las capas lipídicas de las membranas. Como en la *Salmonella* los poliprenolfosfatos están involucrados en la formación de antígenos específicos, parece probable que en los tejidos animales puedan ser los responsables de la formación de los polisacáridos superficiales tan importantes en el comportamiento de las células en contacto. Estas sustancias externas específicas y sus interacciones, que Kalckar [43] en uno de sus penetrantes ensayos llamó "ectobiológicas", parecen ser de gran importancia en el comportamiento "social" de las células. Sin duda este puede convertirse en un problema fascinante para la investigación del futuro. Afortunadamente, aun después de dos décadas nuestro campo de investigación no se ha hecho aburrido o demasiado de moda.

AGRADECIMIENTOS

Mi entera carrera de investigador fue realizada bajo la influencia del doctor Bernardo A. Houssay, quien dirigió mi tesis doctoral y quien, durante todos estos años, me dio generosamente sus inestimables consejos y amistad. También debo mucho a mis amigos, colegas y colaboradores, cuyos nombres se mencionan en el texto.

Agradezco también la ayuda de la Fundación Campomar, Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Exactas y Naturales (UBA), los National Institutes of Health (EE.UU.) y la Fundación Rockefeller, que nos permitió llevar a cabo nuestro trabajo.

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TRABAJOS SELECCIONADOS

BIOCHEMICAL JOURNAL
Vol 33, p. 734, 1939

FATTY ACID OXIDATION IN LIVER

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(Received 7 March 1939)

FATTY acid oxidation in tissues has been studied by measuring the oxygen uptake or the reaction products (acetoacetic and β-hydroxybutyric acids). The quantitative importance of β-oxidation cannot be ascertained in this way, as a fraction of the acids might undergo some other type of oxidation, such as the ω-oxidation of Verkade & van der Lee [1934].

In Knoop's classical theory the successive elimination of a two-carbon substance is required. This substance has been supposed to be acetic acid, but has never been identified.

The tissue slice technique of Warburg has been aplied to the study of this problem by Edson [1935, 1, 2; 1936], Jowett & Quastel [1935, 1, 2, 3], Edson & Leloir [1936], Mazza [1936], Cohen & Stark [1938], etc. Combining this method with microestimations of fatty acids we have endeavoured to obtain more quantitative results for the oxidation of normal fatty acids with 1-8 C atoms.

Whereas liver slices rapidly oxidize fatty acids, ground tissue or enzyme preparations have never shown any such activity. For this reason it has been supposed that fatty acid oxidation is in some way dependent on cell structure. We have found it possible to prepare a cell-free liver "brei" which will oxidize butyric acid, but attempts to isolate the enzyme system have so far failed, presumably owing to a rapid inactivation by reduction of some of the components.

METHODS

Flasks as described by Krebs [1933] were filled with 30 ml. $NaHCO_3$-Ringer solution [Krebs, 1933] and with rat liver slices (about 200 mg. dry wt.). After mixing the contents thoroughy, a 12 ml. initial sample was withdrawn; the flasks were then gassed with O_2+5 % CO_2 and shaken 2 hr. at 37·5°.

Bicarbonate was estimated as previously described [Leloir & Muñoz, 1937].

Ketone bodies. Acetoacetic acid was estimated by both the manometric and modified Van Slyke methods as described by Edson [1935, 1]. The amount of NaOH given by Edson for the Rupp titration is slightly too small and may give rise to errors. It is better to double the amount, and then use a double quantity of acetic acid.

Every estimation was carried out in duplicate, the agreement being good (5 %). The agreement with the manometric method was also good (difference less tran 10 %), but only with amounts larger than 50μl.

Fatty acids. All the existing methods for fatty acid estimation require relatively large amounts of acid. In order to obtain greater accuracy and a shorter time of distillation we have used small volumes. Distillation was carried out after treating the samples with copper-lime reagent, because glucose can give rise to the formation of distillable acid. For estimating acids of 3 to 8 C atoms in the presence of acetic acid, we have taken advantage of the fact that the latter is not oxidized by dichromate. Interfering volatile substances were eliminated by alkaline evaporation in the presence of HgO. Decanoic acid can also be estimated in this way, but it was not used in our experiments because of the insolubility of its Ca salt.

The details of the fatty acid estimations are as follows.

Precipitation of proteins. In experiments with liver slices the liquid can be directly

treated with copper-lime in the amounts given by Edson [1935, 1]. For liver brei, proteins were precipitated with 1 ml. 10 % $ZnSO_4$ per ml. brei and NaOH, the amount of which was ascertained by titrating the zinc sulphate in the presence of phenol red to an orange yellow. The liquid was then diluted 7 times and filtered. Zn cannot be used with octanoic acid as it forms an insoluble salt. Hg and Cu octanoates are also insoluble, but the latter redissolves on adding $Ca(OH)_2$ and recovery is quantitative.

Samples for acidimetric titration were directly distilled, whereas those for dichromate oxidation were treated as follows:

Elimination of interfering substances. The sample (6-8 ml.) after deproteinization was measured into a test tube (15×140 mm.), followed by 0·2 ml. 2·5 *N* NaOH, 0·2-0·3 g. powdered HgO (yellow)) and a small piece of porous porcelain. It was then placed in a boiling salt water bath (105°), the test tube rack being suspended in such a way that the tubes were only partly immersed, so that their boiling could be easily controlled.

Boiling was continued until the samples were evaporated to half volume (about 1-2 hr.), the liquid being then ready for distillation. HgO has also been used by Friedemann [1938] in order to remove aldehydes, formic, pyruvic and crotonic acids, etc.

Distillation. An all glass apparatus similar to that described by Nicloux *et al.* [1934] was used. As it is very important that the rate of distillation should be reproducible and constant, electric heating was used, the column was covered with cotton wool and the flask surrounded by a wide glass tube.

6 ml. of the sample were measured into the distillation flask, followed by 2 g. anhydrous Na_2SO_4, 1 ml. H_2SO_4 (2 vol. conc. H_2SO_4 to 1 vol. H_2O) and a capillary tube to avoid bumping. Crystallization occurs before the end of the distillation if smaller amounts of H_2SO_4 are used, but not under the given conditions. Distillation takes about 15 min. and was interrupted when 5 ml. distillate had collected in a 15×140 mm. pyrex test tube.

Acidimetric titration. The contents of the test tube are boiled in an open flame for 10 sec. in the presence of a small crystal of $BaCl_2$. This removes the CO_2 and detects the presence of H_2SO_4, any trace of which would cause the estimation to be discarded. The solution is then titrated with 0·01 *N* NaOH and phenolphthalein.

Dichromate oxidation. Oxidizing solution: 2·45 g. $K_2Cr_2O_7$ are dissolved in 1 l. of conc. H_2SO_4 (heat until white fumes appear). 10 ml. of this solution are sufficient for oxidizing up to 2 ml. 0·01 *N* hexanoic acid. For the same amount of octanoic acid the solution should contain double the amount of $K_2Cr_2O_7$.

To 5 ml. distillate contained in a test tube, 10 ml. of $K_2Cr_2O_7$-H_2SO_4 are added. The liquid is allowed to fall directly on the surface of the distillate so that immediate mixing occurs. A blank with distilled water is run at the same time. The tubes are covered with a small beaker and then immersed in a boiling water bath for 1 hr. The contents of the tubes are then quantitatively transferred into a 250 ml. Erlenmeyer flask, using about 100 ml. water. After adding 1 ml. 10 % KI the liberated I_2 is titrated with 0·025 *N* $Na_2S_2O_3$.

Calculation. The ml. thiosulphate used in titrating the blank minus those used for the unknown are multiplied by 2·5×22·4 and divided by the oxidation equivalent (Table I). This gives the amount of fatty acid in μl.

The results obtained by applying these methods to pure solutions are shown in Table I. 95 % of the acetic acid is recovered after distillation, and recovery is quantitative for the other acids within the titration error. Results of the $K_2Cr_2O_7$ oxidation show errors not exceeding 10 %, which is satisfactory for work with liver slices. There are some differences in the oxidation equivalents, these errors being specially due to the acidimetric titration. With small amounts of acids this error becomes greater; and with higher fatty acids which are insoluble in water, the formation of the Na salt takes some time and requires strong shaking. This explains the too high value obtained in the oxidation equivalent when 2 ml. of octanoic acid were used (Table I).

Table I. *Acidimetric and oxidimetric estimations of fatty acid solutions*

Acid solution	Titration with 0·01 *N* NaOH (ml.)		Titration with 0·01 *N* $Na_2S_2O_3$ (ml.)		ml. $Na_2S_2O_3$ / ml. NaOH
ml.	Direct	Distilled	Direct	Distilled	
			Acetic		
1·0	0·975	0·924	—	—	—
1·0	0·969	0·935	—	—	—
2·0	1·908	1·862	—	—	—
2·0	1·910	1·797	—	—	—
			Propionic		
0·5	0·465	0·431	4·86	5·11	—
0·5	0·461	0·424	5·04	5·08	11·9
1·0	0·922	0·875	9·80	10·10	—
1·0	0·925	0·865	9·90	9·75	11·4
2·0	1·775	1·695	19·80	19·95	—
2·0	1·790	1·735	20·00	19·60	11·5
			Butyric		
0·5	0·438	0·455	8·10	8·56	—
0·5	0·443	0·473	8·09	8·32	17·3
1·0	0·918	0·932	15·93	16·10	—
1·0	0·927	0·948	15·98	15·98	17·1
2·0	1·850	1·859	31·50	31·80	—
2·0	1·854	1·870	31·55	31·20	16·9
			Valeric		
0·5	—	0·461	—	11·04	—
0·5	—	0·458	—	10·91	23·9
1·0	0·985	0·956	21·83	21·67	—
1·0	0·991	0·935	21·40	21·51	22·8
2·0	—	1·875	—	42·50	—
2·0	—	1·862	—	42·40	23·8
			Hexanoic		
0·5	—	0·430	—	11·13	—
0·5	—	0·454	—	11·40	25·6
1·0	—	0·920	—	22·65	—
1·0	—	0·938	—	22·65	24·65
2·0	—	1·830	—	43·80	—
2·0	—	1·860	—	43·20	23·6
			Heptanoic		
0·5	—	0·432	—	14·30	—
0·5	—	0·455	—	13·80	32·0
1·0	—	0·900	—	26·30	—
1·0	—	0·894	—	26·70	29·5
2·0	—	1·800	—	52·60	—
2·0	—	1·760	—	51·70	29·2
			Octanoic		
0·5	—	0·413	—	18·0	—
0·5	—	0·420	—	17·63	42·7
1·0	—	0·795	—	32·9	—
1·0	—	0·787	—	34·0	42·4
2·0	—	1·483	—	65·9	—
2·0	—	1·438	—	—	45·0

Formic acid. Distillation under the described conditions is not quantitative (about 70 %), therefore estimations with $HgCl_2$ were carried out on the samples after copper-lime treatment. The method was used as described by Riesser [1915] but with smaller amounts. In a test tube with a ground glass stopper, 5 ml. of the filtrate were carefully neutralized (phenol red), and 1 ml. of the $HgCl_2$ reagent added ($HgCl_2$ 300 g., Na acetate 300 g., NaCl 80 g., per l.). The tubes were then heated in a salt water bath (105°) for 40 min. After cooling, 0·5 ml. glacial acetic acid, 1 ml. saturated KI and 2 ml. 0·03 *N* I_2 were added. The tubes were shaken, and after complete solution of the calomel the excess I_2 was titrated with 0·01 *N* $Na_2S_2O_3$.

Table II. *Liver slices from rats starved 24 hr.*

Bicarbonate Ringer. Gas O_2+5 % CO_2. △ indicates the difference in composition (in μl.) of 1 ml. medium before and after 2 hr. at 37.5°

No	Volume of medium ml.	Dry wt. of slices (mg.)	Substrate	Bicarbonate △	Bicarbonate Q	Acid by oxidation △	Acid by oxidation Q	Distillable acid △	Distillable acid Q	Acetoacetic acid △ Titr.	Acetoacetic acid △ Manom.	β-Hydroxy-butyric acid △	Total ketonic acids △	Total ketonic acids Q
1	18.0	188	Formate 0.0118 *M*	− 20	− 0.96	− 23	− 1.1	—	—	25	43	21	46	+ 2.2
	18.0	170	None	− 64	− 3.40	+ 24	+ 1.27	—	—	27	33	23	50	+ 2.65
2	17.4	276	Formate 0.008 *M*	− 26	− 0.82	− 2	− 0.06	—	—	6	8	7	13	+ 0.44
	17.4	278	None	− 47	− 1.47	+ 24	+ 0.75	—	—	4	9	8	12	+ 0.38
3	18.8	254	Acetate 0.0136 *M*	+ 75	− 2.8	—	—	− 144	− 5.3	12	18	20	32	+ 1.2
	18.8	234	None	− 45	− 1.81	—	—	0	0	3	—	7	10	+ 0.4
4	20.8	246	Acetate 0.0107 *M*	+ 55	− 2.3	—	—	− 91	− 3.8	26	29	12	38	+ 1.7
	20.8	219	None	− 26	− 1.2	—	—	0	0	7	6	16	23	+ 1.1
5	20.8	228	Acetate 0.0284 M	+ 49	− 2.2	—	—	− 112	− 5.1	46	51	19	65	+ 3.0
	20.8	222	None	− 44	− 2.2			0	0	13	20	13	26	+ 1.2
6	18.0	235	Propionate 0.0125 *M*	− 15	− 0.57	− 58	− 2.22	− 46	− 1.80	5	9	9	14	+ 0.54
	18.0	251	None	− 25	− 0.90	0	0	0	0	3	4	7	9	+ 0.25
7	18.0	273	Propionate 0.01 *M*	− 32	− 1.0	− 27	− 0.89	− 9	− 0.33	12	21	13	25	+ 0.84
	18.0	270	None	− 37	− 1.23	0	0	0	0	5	11	12	16	+ 0.57
8	18.0	184	Butyrate 0.0135 *M*	− 14	− 0.7	199	− 9.8	− 175	− 8.6	100	100	56	155	+ 7.6
	18.0	191	None	− 26	− 1.2	0	0	0	0	4	12	10	14	+ 0.7
9	18.0	226	Butyrate 0.0133 *M*	− 24	− 0.9	− 242	− 9.7	− 237	− 9.5	158	157	71	229	+ 9.1
	18.0	198	None	− 19	− 0.9	0	0	0	0	+ 8	+ 10	+ 10	+ 17	+ 0.5
10	19.0	206	Butyrate 0.0177 *M*	− 12	− 0.6	− 167	− 7.7	− 187	− 8.6	119	120	65	184	+ 8.5
	19.0	188	None	− 29	− 1.5	0	0	0	0	4	10	1	5	+ 0.3
11	17.4	292	Valerate 0.0090 *M*	− 80	− 2.38	− 68	− 2.03	− 47	− 1.40	6	17	17	24	+ 0.71
	17.4	245	None	− 72	− 2.57	0	0	0	0	4	8	13	17	+ 0.60

12	17·4	244	Valerate 0·0086 *M*	— 52	— 1·86	— 2·25	— 63	— 52	— 1·86	19	24	19	38	+ 1·36
	17·4	250	None	— 42	— 1·46	0	0	0	0	6	10	10	16	+ 0·56
13	17·4	190	Hexanoate 0·0087 *M*	— 97	— 4·45	— 164	— 7·55	— 137	— 6·28	144	147	99	243	11·1
	17·4	195	None	— 35	— 1·56	0	0	0	0	24	29	18	42	1·82
14	18·0	151	Hexanoate 0·0121 *M*	— 61	— 3·63	— 97	— 5·8	— 81	— 4·8	102	107	39	+ 141	+ 8·4
	18·0	178	None	— 43	— 2·17	0	0	0	0	25	32	15	+ 40	+ 2·0
15	18·0	226	Hexanoate 0·0117 *M*	— 64	— 2·55	— 120	— 4·77	— 125	— 4·97	87	89	84	+ 170	+ 6·37
	18·0	233	None	— 48	— 1·85	0	0	0	0	4	9	10	+ 14	+ 0·54
16	17·4	201	Heptanoate 0·0095 *M*	— 49	—'2·12	— 65	— 2·81	— 42	— 1·86	22	28	15	38	+ 1·64
	17·4	228	None	— 72	—'2·72	0	0	0	0	22	29	11	33	+ 1·26
17	17·4	256	Heptanoate 0·0103 *M*	— 95	—'3·23	— 91	— 3·1	— 23	— 0·78	13	21	23	36	+ 1·22
	17·4	244	None	—'56	— 2·00	0	0	0	0	6	13	10	16	+ 0·57
18	17·4	213	Octanoate 0·01 *M*	— 138	— 5·6	— 156	— 6·4	— 110	— 4·5	+ 106	+ 111	+ 92	+ 198	+ 8·1
	17·4	175	None	— 51	—'2·7	0	0	0	0	4	8	4	8	0·4
19	17·4	160	Octanoate 0·0089 *M*	— 174	—'9·4	— 131	— 7·1	— 88	— 4·8	+ 112	+ 116	+ 122	+ 234	+ 12·8
	17·4	114	None	— 86	—'4·7	0	0	0	0	9	17	+ 6	+ 15	+ 0·8
20	17·4	196	Octanoate 0·0096 *M*	— 116	— 5·2	— 155	— 6·9	— 96	— 4·3	+ 144	+ 137	+ 87	+'231	+ 10·2
	17·4	246	None	— 44	— 1·6	0	0	0	0	+ 25	+ 20	+ 14	+ 38	+ 1·3
21	17·4	234	Octanoate 0·0090 *M*	— 154	—'5·7	— 144	— 5·4	— 98	— 3·7	+ 73	+ 74	+ 165	+ 238	+'8·9
	17·4	230	None	— 55	—'2·1	0	0	0	0	+ 5	+ 10	+ 12	+ 18	+ 0·7
22	17·5	244	Octanoate 0·008 *M*	— 123	— 4·42	— 121	— 4·34	— 70	— 2·51	45	49	133	178	+ 6·39
	17·5	269	Acetate 0·022 *M*	+ 121	+ 3·96	0	0	— 205	— 6·69	14	23	16	30	+'0·98
23	17·5	221	Octanoate 0·095 *M*	— 94	— 3·72	— 117	— 4·64	— 88	— 3·49	63	68	115	178	+ 7·05
	17·5	236	Acetate 0·0125 *M*	+ 90	+ 3·34	0	0	— 125	— 4·64	7	—	8	15	+ 0·56
24	17·5	237	Octanoate 0·0079 *M*	— 97	— 3·58	— 128	— 4·72	— 71	— 2·62	98	100	116	215	+ 7·87
	17·5	244	Acetate 0·0125 *M*	—	—	0	0	— 134	— 4·81	15	22	15	30	+ 1·08

With pure solutions the results are reproducible within 10 % with amounts ranging from 30 to 300μl. (0·06-0·6 mg.).

Units. Results are given in μl., the acids being considered as perfect gases at N.T.P.[1] (22·4μl.=1μmol.). Q represents μl. of substance formed per mg. tissue (dry wt.) per hr.

EXPERIMENTAL RESULTS

One of the difficulties in the interpretation of the results is that in the control there is always a spontaneous formation of ketonic acids and that it is impossible to know if this continues at the same rate when a substrate is added. This also applies to the measurements of Na HCO_3. Liver slices with no substrate produce a decrease in $NaHCO_3$, less than half of which is due to ketonic acids. The rest is not due to lactic acid or to a distillable acid. Perhaps it is due to a fixation of base (K) by the liver slices.

For this reason we shall often refer to the corrected Q. This is the value obtained by subtracting the value of Q given by a control with no substrate. Measuring as we have done in every case the distillable acid, acetoacetic and β-hydroxybutyric acids and $NaHCO_3$, we can get a rough idea of the formation of a non-distillable non-ketonic acid.

Slices in the presence of, e.g., Na butyrate, consume the butyrate ion and an increase in $NaHCO_3$ occurs; ketonic acids are formed decreasing the $NaHCO_3$, and if any other acid is formed it will also decrease $NaHCO_3$. We should then have:

$$-Q_{\text{bic.}}\,(\text{corr.}) = Q_{\text{dist. ac.}} + Q_{\text{ketonic ac.}} + Q_{NN}.$$

Q_{NN} would therefore represent the non-distillable non-ketonic acid. Naturally, as this is calculated indirectly, Q_{NN} will only be significant when its value is large.

Formic acid. Liver slices without substrate give rise to the formation of a substance which is estimated as formic acid (see Table II, Nos. 1 and 2). This amounts to about 24μl. per ml., giving a Q_{formic} of 1·27 and 0·75. The method of estimation used is far from specific and we cannot assert that this substance is really formic acid.

On adding formic acid to liver slices a small disappearance occurs: $Q_{\text{formic}} = -1{\cdot}1$ and $-0{\cdot}06$. Subtracting the spontaneous formation, the values for the disappearance ($-Q$) would be 2;37 and 0·81 respectively. Ketonic acid formation is not modified and the acid disappearance is in good agreement with the changes in $NaHCO_3$: Q_{bic}. (corrected) 2·44 and 0·65. The velocity of disappearance of formic acid is therefore small, and if it were formed from added fatty acids we should expect it to accumulate to a certain extent in the medium. As we shall see later, this is not the case.

Acetic acid. Acetic acid disappears at a rate more than twice that of formic acid.

The values of $-Q_{\text{acetic}}$ obtained by distillation and titration with NaOH were 5·3, 3·8 and 5;1; corresponding $Q_{\text{bic.}}$, 4·6, 3·5 and 4;4. Q_{keto}. (corrected) amounted to 0·8, 0·6 and 1·8.

Calculating with these results the non-distillable, non-ketonic acid ($Q_{NN} = -Q_{\text{bic}} - Q_{\text{dist.}} - Q_{\text{keto.}}$) we obtain $-0{\cdot}1$, $-0{\cdot}3$ and $-1{\cdot}1$. Therefore, when acetic acid disappears, there is no formation of any other acid except acetoacetic and β-hydroxybutyric.

It is clear from these experiments that the increase in ketonic acids only accounts for a small fraction of the acetic acid which disappears. The amount of acetic acid which disappears is 6·6, 6·3 and 2·8 times greater than the ketonic acids formed (mol. per mol.).

The mechanism of this reaction has been discussed by Krebb & Johnson [1937]. They give good evidence that the first step is a condensation of acetic with pyruvic acid, acetopyruvic acid being formed. The latter is then transformed into ketonic acids.

Acetic acid increases Q_{O_2} by 2·4 units and is therefore probably oxidized. If the oxidation were direct, the only possible-intermediary would be glycollic acid, which would then be oxidized to glyoxylic and this acid might give 2 mol. of formic acid or be oxidized to oxalic acid. But this does not occur in liver, as is proved by the experiment in Table III in which the changes in bicarbonate and ketonic acids were measured.

[1] We have continued using μl. because it is the unit used by all those who have worked with tissue slices, but it would be more correct to use μmol.

TABLE III *Liver slices in bicarbonate Ringer*

	Q_{bic}	Q_{keto}
No substrate	— 1·30	0·34
Acetate 0·02 *M*	+ 2·69	1·38
Glycollate 0·002 *M*	— 2·92	0·19
Oxalate 0·02 *M*	— 1·23	0·78

This experiment shows that the acetate ion disappears, producing an increase in base (Q_{bic} ·). In the presence of glycollate this increase in base does not occur; on the contrary there is a slight acidification which might be due to oxidation to oxalate. Oxalic acid is not oxidized, for this were the case it would give two basic equiv. per mol.

In another identical experiment formic acid was also estimated, no difference being found between the flask with no substrate and that with glycollic acid.

If acetic acid disappears by condensation with another substance one would expect that the addition of that substance would increase the rate of disappearance. Experiments in this direction were not quite satisfactory, because our method was not capable of detecting very small changes. Nevertheless we have tried many substances (C_4 dicarboxylic acids, glycine, aspartic acid, insulin, dry thyroid, glucose, fructose, lactante, citrate, liver and yeast extracts etc.) without finding any appreciable increase in the rate of disappearace.

Malonic acid inhibits acetic acid disappearance (*M*/50 malonate decreases the $-Q_{acetic}$ from 5·1 to 2·1).

Propionic acid. The rate of disappearance is small ($Q_{propionic}$ = − 2·22 and − 0·89); decrease in distillable acid, − 1·80 and − 0·33. Q'_{bic}. (corrected) = 0·33 and 0·23: increase in ketonic acids = 0·29 and 0·27.

As propionic acid is metabolized slowly we have not tried to determine what is the first reaction product.

Butyric ocid. Of all the acids studied butyric is oxidized most rapidly ($-Q'_{butyric}$ = 9·8, 9·7 and 7·7, Table II). The corresponding values of Q_{keto} . were 6·9, 8·6 and 8·2. Therefore 70, 89 and 106 % of the butyric acid was transformed into ketonic acids.

The values of Q_{bic} . (corrected) were 0·5, 0 and 0·9. This shows that only a small amount is totally oxidized (5, 0 and 12 % respectively).

Valeric acid. Experiment with valeric acid (Nos. 11 and 12, Table II) gave the following results: $Q_{valerie}$ = − 2·03 and − 2·25. $Q_{dist.\ ac.}$ = − 1·40 and − 1.86. Q_{bic}· (corrected) = + 0·2 and − 0·40. Q_{keto} (corrected) = 0·11 and 0·8. The difference between the values obtained by titration with NaOH and by oxidation are too small to be significant. The amount of non-distillable non-ketonic acid would be 1·09 and 1·46; values to which no importance can be given owing to the indirect way in which they are calculated.

Hexanoic. The rate of disappearance of hexanoic acid, as measured by the oxidation method, was $-Qhexanoic$ = 7.55, 5.8 and 4.77; and as measured by distillation and titration with NaOH: $-Q_{dist.\ ac.}$ = 6 28, 4.8 and 4.97. The difference between these values (1.27, 1.0 and 0) is attributed to a small accumulation of acetic acid.

If we suppose that each molecule of hexanoic gives rise to one of ketonic acid and one of acetic, the $Q_{ketonic\ ac.}$ should be equal to the $Q_{hexanoic}$ plus the amounts of ketonic acids which are formed from acetic acid.

The values found for the $Q_{ketonic\ ac.}$ (corrected) were 9.28, 6.4 and 5.83. They are larger than the $Q_{hexanoic}$, the excess being: 1.7, 0.6 and 1.06. These values are of the order of those found for acetic acid which can increase the $Q_{\textbf{ketonic ac.}}$. by 1 or 2 units. Moreover, the amount of acetic acid formed should be equal to the $-Q_{hexanoic}$. Of this, part accumulates in the medium (1.27, 1.0 and 0) and the rest (6.28, 4.8 and 4.97) would disappear. The latter values are of the order of those found for the disappearance of added acetic acid.

The values of $-Q_{bic}$. (corrected) were 2.89, 1.46 and 0.80. From these we can calculate the non distillable non-ketonic acid (− 0.11, − 0.14 and − 0.06). These small values not only show that no fixed acid is formed but also that there is a good agreement between the different methods of estimation.

Heptanoic (Exps. 15 & 16, Table II). Values obtained for the disappearance of heptanoic were $-Q_{heptanoic} = 2.81$ and 3.1; $-Q_{dist.\ ac.} = 1.86$ and 0.78. The difference between these values (0.95 and 2.32) would indicate the accumulation of a distillable acid which is not oxidized with dichromate (acetic).

The increases in the $Q_{ketonic\ ac.}$ were 0.38 and 0.65. The $Q_{bic.}$ (corrected) $= +0.60$ and -1.23. The non-distillable non-ketonic acid would be 0:88 and 1:36. All these results may be interpreted by the classical β-oxidation: 2 mol. acetic acid and 1 mol. propionic acid being formed from each mol. of heptanoic acid.

The lanthanum reaction, using the technique described for octanoic acid, was carried out in three experiments. The final sample of the flask containing slices and heptanoic acid gave a positive reaction. The reaction loses in this case some of its value because the positive result could be due to propionic acid.

Octanoic acid. This acid disappears at a greater rate than any of the odd numbered acids. $-Q_{octanoic} = 6.4$, 7.1, 6.9 and 5.4. The corresponding $Q_{ketonic\ ac.}$ (corrected) 7.7, 12, 8.9 and 8.2. Therefore each mol. octanoic acid gives rise to 1.2, 1.7, 1.3 and 1.5 mol. ketonic acid (Exps. 18, 19, 20 and 21, Table II).

The amount of acetic acid formed would be ($Q_{acetic} = Q_{octanoic} - Q_{dist.\ ac.}$) 1.9, 2.3, 2.6 and 1.7. The $Q_{bic.}$ values (corrected) were: -2.9, -4.7, -3.6 and -3.6.

The calculation of the amount of non-distillable non-ketonic acid gives negative values (-0.3, -2.5, -1.0 and -0.9).

According to the classical β-oxidation each mol. octanoic acid should give one of ketonic acid and two of acetic. Therefore $Q_{ketonic}$ should be equal to the $-Q_{octanoic}$ and double this amount of acetic should be formed. But in our experiments the $Q_{ketonic\ ac.}$ exceeds the $Q_{octanoic}$ by 1.3, 4.9, 2.0 and 2.8. Some of these values are considerably greater than the amount of ketonic acids that arise from acetic acid and cannot be attributed to experimental errors as every estimation was carried out in duplicate. Moreover, the amount of acetic acid formed should be double the $-Q_{octanoic}$, that is 12.8, 14.2, 13.8 and 10.8; of this a part ($Q =$ about 2) accumulates in the medium and the rest should disappear. But we have seen that acetic disappears at most at a rate of $Q = 4$–6. If we suppose that each mol. of octanoic acid is split into two of ketonic acid the $Q_{ketonic}$ should be double the $Q_{octanoic}$ and this does not explain the experimental results.

We may then suppose that octanoic acid can be oxidized by both mechanisms, a fraction (a) would give 2 mol. ketonic acid and the rest (b) would give 1 mol. ketonic acid and 2 mol. acetic acid.

To test this hypothesis we carried out another set of experiments (Nos. 22, 23 and 24) in which acetate was added to the control, enabling us to ascertain for the liver specimen how much acetate disappears and the amount of ketonic acids which are formed from it.

The amount of octanoic acid which is oxidized by the mechanisms (a) and (b) is calculated as follows: $Q_{octanoic} = a + b$ (I). The amount of ketonic acids formed will be (II) $2a + b + Q_{ketonic}$ (control) (this represents the amount formed spontaneously and from acetic acid).

Replacing in (II) the value of (b) in (I) we obtain:

$$a = Q_{ketonic} - Q_{octanoic} - Q_{ketonic}\ \text{(control)}$$

On applying this equation to the experimental results we obtain the values given in Table IV.

Table IV

$-Q_{octanoic}$	a	b	$\frac{b}{-Q_{octanoic}}$
4.34	1.07	3.27	0.75
4.64	1.85	2.79	0.60
4.72	2.07	2.65	0.56

We can now calculate the amount (2*b*) of acetic acid formed from octanoic acid. We know by experiment how much acetic acid accumulates and we also know the rate at which acetic acid disappears in that liver (from the control). We can then compare the values for acetic acid formation calculated from the Q_{octanoic} and the Q_{ketonic} with the values obtained from the accumulated acetic acid: ($Q_{\text{dist. ac.}} - Q_{\text{octanoic}}$) plus the acetic acid which disappears in the control.

The $-Q_{\text{acetic}}$ of the control = 6.69, 4.64 and 4.81. Accumulated acetic = 1.83, 1.15 and 2.10. The sum of these values would be the acetic acid formed from octanoic = 8.5, 5.7 and 6.9 (calc. values (2*b*) = 6.5 ,5.6 and 5.3). The agreement is good if we consider that the acetic acid formed is calculated in an indirect way.

Acetic acid formation from octanoic acid. In the experiments with octanoic acid we have found a difference of 1 or 2 units in the Q as measured by oxidation and as measured by titration with NaOH. This we have attributed to an accumulation of acetic acid. These results may also be attributed to the formation of acids with less C atoms (formic, butyric or hexanoic).

Formic acid formation is excluded because estimations showed no difference between slices with and without octanoic acid. Butyric acid disappears faster than octanoic so that its accumulation is not probable.

Lanthanum reaction. In order to confirm the fact that acetic acid is really formed we hace used the lanthanum reaction. Acetic acid does not give the lanthanum reaction in the presence of octanoic acid, which must be removed as its less soluble Ag salt before carrying out the test.

The liquid to be examined was treated with copper-lime, the alkaline filtrate was evaporated to dryness in a water bath; the residue was extracted with hot water (6 mil.) and distilled. The distillate (5 ml.) was carefully neutralized, solid Ag_2SO_4 added and the solution was boiled and then cooled to $-2°$ and filtered. The filtrate was distilled and the lanthanum reaction [Krüger & Tschirch, 1930] applied to the distillate.

As in all the experiments, liver slices (200 mg. dry wt.) were suspended in 30 ml. of $NaHCO_3$-Ringer in two flasks. One of them contained octanoate (0.01 *M*) and the other no substrate. An initial sample of 12 ml. was withdrawn from each and the rest left 2 hr. at 37°. Of these four samples treated in the same manner only one gave a positive lanthanum reaction. This corresponded to the final sample of the flask with octanoate.

That acetic acid responsible for the positive lanthanum reaction does not arise from the action of alkali on acetoacetic acid was proved by adding β-hydroxybutyric acid to some of the controls. Although a considerable formation of acetoacetic acid occurred, the La reaction was always negative.

Oxidation of butyric acid in a cell-free "brei"

Preparation of the "brei". We have used a similar procedure to that described by Potter & Elvehjem [1936]. A bulb is blown at the end of a capillary tube in such a way that it fits with less than 0:3 mm. clearance in a strong test tube (16 × 150 mm. or 22 × 200 mm. according to the amount of tissue). The liver of one or two recently killed rats is weighed, cut in small portions with scissors and put in the test tube which already contains the cooled alkaline buffer (31 g. $Na_2HPO_4, 2H_2O$ + 8 g. KCl per l.; 1.53 ml. per g. liver). With the test tube in a freezing mixture, the piston is introduced and worked up and down energetically. When the contents are frozen the operation is continued out of the freezing mixture. After thawing, the tube is again put in the freezing mixture. The procedure is continued for 10 min., 0.5 *M* KH_2PO_4 (0.87 ml. per 10 ml. of alkaline buffer) is added and the liquid is filtered through muslin. During all these manipulations the contents of the tube are aerated with O_2.

Microscopical examination [1] of the "brei" so obtained shows the absence of liver cells, only some nuclei and white and red blood cells being visible. (Blood has no oxidative activity.) The resulting *p*H is optimal for the preparation, addition of small amounts of acid or alkaline buffer decreasing the activity.

Activity measurements can only be made by

[1] We are indebted to Dr. Porto for the microscopical study of our preparations.

estimating butyric acid. The O_2 uptake without added substrate is so great that it cannot be used to determine the rate of butyric acid oxidation.

Butyric acid added to this "brei" at 25° in O_2 disappears. That this disappearance is due to oxidation is proved by estimation of acetoacetic and β-hydroxybutyric acids. The "brei" is rapidly inactivated in absence of O_2: it is sufficient to leave it in a test tube at room temperature for 15–30 min. without bubbling O_2 through it, in order to obtain complete inactivation.

Succinic, fumaric, malic and citric acids added to the "brei" increase the disappearance of butyric acid but exert no action once the "brei" has been inactivated by anaerobiosis.

Table V. *Cell-free liver "brei" 7 ml. (about 700 mg. dry wt.). 2 hr. at 25°. Gas O_2*

	Butyric μl.	Δ μl.	Aceto-acetic acid μl.	β-Hydroxy-butyric acid	Total ketonic acid μl.	Ketonic Butyric
Butyrate added	3029	—	—	—	—	—
"Brei" + butyrate	2370	−742	788	420	+796	1:07
„ + no substrate	83	—	137	412	—	—
„ + butyrate + fumarate	2159	−924	125	835	+640	0:69
„ + fumarate (0:01 *M*)	74	—	5	195	—	—

In Table V we give the results of one of three exp. with fumaric acid. It shows that the "brei" with no substrate forms a certain amount of ketonic acids. When butyric is added it disappears and there is a corresponding increase in total ketonic acids. When both butyric and fumaric acids are added more butyric disappears (sometimes 50 % or more), but the ketonic acid formation does not increase so much. With butyric acid alone the relation $\frac{\text{total ketonic}}{\text{bytyric acid}}$ was 1.07 and when fumaric was also present it was only 0.69.

Moreover, when fumaric is present more of the ketonic acid appears in the reduced state. The relation $\frac{\beta\text{-hydroxybutyric}}{\text{acetoacetic}}$ is 0.5 with butyric acid alone and 5.3 when fumaric acid is also present.

Discussion

The methods described are suitable for the type of experiments for which we have used them, and with slight modifications might be useful for other purposes. They are good for acids with 3–8 C atoms, the oxidation with dichromate being more accurate than acidimetric titration. One important point is that acetic acid does not interfere in the oxidation method.

Measurements of the rate of disappearance of saturated fatty acids show a net difference between the odd and even series. This difference is also observed in the ketogenesis, but is not clear from measurements of O_2 uptake: the increases in Qo_2 [data of Edson, 1935, 1] being C_1, 1.1; C_2, 3.8; C_3, 0.6; C_4, 4.0; C_5, 2.1; C_6, 1.7; C_7, 2.4; C_8, 1.3. By measurement of the disappearance of the acids we have obtained C_1, 1.5; C_2, 5; C_3, 2; C_4, 9; C_5, 2; C_6, 6; C_7, 3; C_8, 6.

Formic acid is presumably oxidized completely to CO_2 and H_2O. Liver slices without any added substrate give rise to a small amount of a substance which is estimated as formic acid (as was observed in liver perfusion by Toenniessen & Brinkmann [1938]). Owing to the lack of specificity of the method we have used we cannot be sure if it is in fact formic acid. The formation of this substance does not increase in the presence of octanoate.

Acetic acid disappears at a much higher rate and, as bicarbonate increases proportionally, we may deduce that no other acid accumulates. Oxalic and glycollic acids do not give an increase in base; therefore they cannot be intermediaries in the disappearance; formic acid can also be excluded as its oxidation is too slow.

The amount of ketonic acids formed from acetic acid only accounts for about 20 % of that disappearing (mol. per mol.). We have not been able to find out how the rest of the

acetic acid is metabolized. If it were by condensation with another substance we might expect an increased disappearance on adding that substance but this was not observed in our experiments. The fact that there is an increase in O_2 uptake with acetic acid indicates that a part of it is oxidized, although probably not directly.

The rate of disappearance of propionic acid is low and therefore we have not tried to find out how it is oxidized.

Butyric is the acid which is most rapidly oxidized by the liver. Most of it (80 or 90 %) is β-oxidized, but we cannot completely dismiss the possibility of a very small fraction undergoing some other type of change.

Valeric disappears at about the same rate as propionic acid. The small increase in ketonic acid formation already observed by Edson [1935, 1] and Jowett & Quastel [1935, 2] might arise from the acetic acid formed. Results obtained with hexanoic and heptanoic acids can be quite well interpreted by β-oxidation.

With octanoic acid, results are rather more complicated. This acid gives more acetoacetic + β-hydroxybutyric than is required by a successive β-oxidation, but not enough to account for the molecule breaking up into two 4 carbon units. If we suppose that a fraction of octanoic acid follows each of these possibilities, the experimental results can be well interpreted.

This type of oxidation of fatty acids has previously been suggested by Jowett & Quastel [1935, 2], although the theory was supported by somewhat indirect evidence. According to Jowett & Quastel the fatty acid molecule (e.g. octanoic) would undergo a simultaneous oxidation at the 2, 4 and 6 C atoms. The triketo-acid formed, which might only exist in combination with the enzyme, can then break down, giving 2 mol. acetoacetic or 1 mol. of acetoacetic and 2 mol. of acetic acid. This interesting hypothesis explains satisfactorily the results we have obtained. In our experiments about 30 % of the octanoic acid would be split into 2 mol. acetoacetic. Moreover, Butts *et al.* [1935] and Deuel *et al.* [1936] have found that feeding rats with measured amounts of the salts or the ethyl esters of hexanoic to tetradecanoic acids leads to the elimination of twice the expected amount of ketonic acids.

When octanoic acid is oxidized by liver slices, a certain amount of acetic acid accumulates. This was ascertained by estimation and by the lanthanum reaction.

Attempts to isolate the enzyme system which oxidizes butyric acid have failed. Precipitates obtained with acetone, ammonium sulphate and acetic acid are inactive and are not activated by adding "kochsaft" or "brei" from liver or muscle. As the inactivation is presumably due to a reduction by the substrates present in the liver, we have also investigated if the presence of oxidants would activate the preparation. However, H_2O_2, ferricyanide, iodate, quinone etc., all acted as inhibitors, and in fact nearly everything tried acted as an inhibitor.

The activating effect of dicarboxylic acids on the "brei" is difficult to understand. Szent-Györgyi [1937] has observed a similar action on the O_2 uptake of pigeon muscle "brei", fumarate acting as an activator but having no action if added after a certain time.

The anaerobic inactivation of the "brei" appears in our case to be the inverse of what is known to occur with other enzymes. Thus papain, cathepsin and succinic dehydrogenase [Hopkins *et al.* 1938] are inactivated by mild oxidizing agents.

Summary

A micromethod for the estimation of fatty acids by distillation and oxidation with dichromate is described.

The action of liver slices on normal fatty acids with 1–8 C atoms was studied.

The rates of disappearance ($-Q$) of the different acids are: formic 1-5; acetic, 5; propionic, 2; butyric, 9; valeric, 2; hexanoic, 6; heptanoic, 3; octanoic, 6.

Glycollic and oxalic acids are not intermediaries in acetic acid disappearance. The ketonic acids formed only account for about 20 % of the acetic acid consumed. Butyric acid is almost completely (80–90 %) oxidized in the β-position.

Hexanoic and heptanoic acids also seem to follow classical β-oxidation.

Octanoic acid appears to be oxidized, a part giving 2 mol. of ketonic acid and another part giving 2 mol. of acetic and 1 mol. of ketonic acid. Acetic acid was identified by the lanthanum reaction.

Butyric acid oxidation can be obtained in a cell-free liver "brei". This preparation is rapidly inactivated, especially under anaerobic conditions.

C_4 dicarboxylic acids appear to exert an activating action on the "brei". They decrease the amount of total ketonic acids formed and increase the reduction of acetoacetic acid.

We wish to thank Prof. B. A. Houssay for his interest and advice in our work and Dr. M. Dixon for kindly correcting the proofs.

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NATURE
Vol. 144, p. 980, 1939

HYPERTENSIN: THE SUBSTANCE CAUSING RENAL HYPERTENSION

An increase in blood pressure is produced by compression of the renal artery [1] or by injection of the venous blood of the kidneys [2]. The filtrate obtained after adding 3 vol. of acetone to the serum of this blood contains a pressor substance which is insoluble in ether and amyl alcohol, soluble in glacial acetic acid and is destroyed only after three hours boiling in normal hydrochloric acid. The same substance is formed on incubating for fifteen minutes at 37° the kidney protein renin [3] with blood serum or its pseudo-globulin fraction. This substance, which we name hypertensin, is different from adrenalin, tyramin, pitressin and urohypertensin. Renin appears to be a proteolitic enzyme of the papain type, which liberates hypertensin from a blood protein belonging to the pseudo-globulin fraction.

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ARCHIVES OF BIOCHEMISTRY
Vol. 18, p. 201, 1948

LETTER (S) TO THE EDITORS

A COENZYME FOR PHOSPHOGLUCOMUTASE

A study of the enzymatic transformation of galactose-1-phosphate revealed that extracts of *Saccharomyces fragilis* will transform this substance into a reducing ester only in the presence of a thermostable factor. The same factor has been found to be necessary for the conversion of glucose-1-phosphate into glucose-6-phosphate with extracts of *S. fragilis* or *S. cerevisiae*.

The action of this coenzyme can be revealed in crude maceration extracts, but these retain a considerable activity in the absence of added coenzyme. Results recorded in Fig. 1 were obtained with a brewers yeast enzyme partially purified by ammonium sulphate fractionation and dialysis. The coenzyme preparation was obtained from brewers' yeast, the ratio: activity/extinction at 260 mμ was 150 times higher than in the extract obtained by heating the yeast in one volume of water and filtering. The ratio activity/total phosphate was 20 times higher.

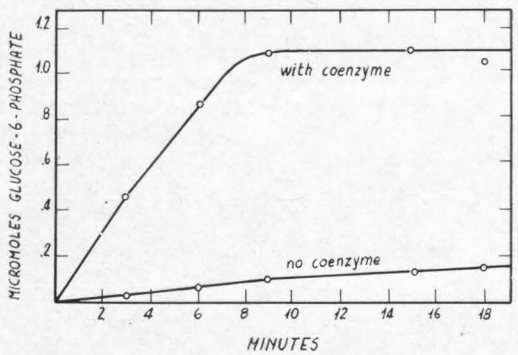

FIG. 1 Activation of phosphoglucomutase with the coenzyme. Reducing power measured with a copper reagent and a glucose-6-phosphate standard. Incubation at 30°C. of: partially purified enzyme, 0.005 μM Mg ++ synthetic glucose-1-phosphate 1.5 μM, purified coenzyme containing 0.1 μM total phosphate. Total volume 0.2 ml.

TABLE I

Changes in Phosphate Fractions

Results in micromoles. Conditions similar to those in Fig. 1. Half the amount of coenzyme.

Coenzyme	Time	P Inorg.	P Labile(*a*)	P Stable(*b*)	Reduction (c)
	min.				
With	0	0.04	0.89	0.07	0.00
With	15	0.08	0.09	0.83	0.86
Without	0	0.05	0.89	0.01	0.00
Without	15	0.08	0.85	0.07	0.01

a Phosphate liberated in 10 min, at 100°C. in 1*N* acid minus inorganic.
b Total phosphate minus labile and inorganic.
c Reducing power in terms of a glucose-6-phosphate standard.

In Table I are recorded the changes in the phosphate fractions and in reducing power. These correspond to those known to be brought about by phosphoglucomutase.

We have been unable to identify this factor with any of the known coenzymes. It can be precipitated from crude solutions with lead, mercury, silver, and barium salts. In general, it follows inorganic phosphate during fractionations with these reagents. Inorganic phosphate can be removed from it as magnesium ammonium or as uranyl salts, when the coenzyme will no longer precipitate with mercury salts.

Purified preparations are colorless and show ultraviolet absorption at 260 mμ, but purification will have to be carried on further to find out whether this absorption is due to the active compound.

Treatment with $N/2$ acid at 100°C. will destroy the activity in 15 min. It is more resistant to treatment with alkali under the same conditions.

Kendal and Stickland (1) obtained an activation of phosphoglucomutase by adding hexose diphosphate, but Cori *et al.* (2) were unable to obtain any effect. The hexosediphosphate preparation of Kendal and Sitckland may have been contaminated with the new coenzyme reported in this paper.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 179, Nº 1, May, 1949, p. 497

THE ENZYMATIC TRANSFORMATION OF GALACTOSE INTO GLUCOSE DERIVATIVES

Sirs:

Extracts of galactose-fermenting yeasts contain the enzyme galactokinase,[1] which catalyzes a transphosphorylation between adenosine triphosphate and galactose. The reaction product galactose-1-phosphate was known to be transformed by crude extracts[2] probaby to glucose-6-phosphate.

A study of this reaction showed that, when a partially purified enzyme of *Sacharomyces fragilis* was used, two additional factors are necessary for maximum activity (Fig. 1). One is thermolabile and the other thermostable. The thermolabile factor is present in muscle and has been identified with phosphoglucomutase by using this enzyme as purified by Najjar,[4] or yeast extract plus glucose diphosphate.[3,5] The reaction would be

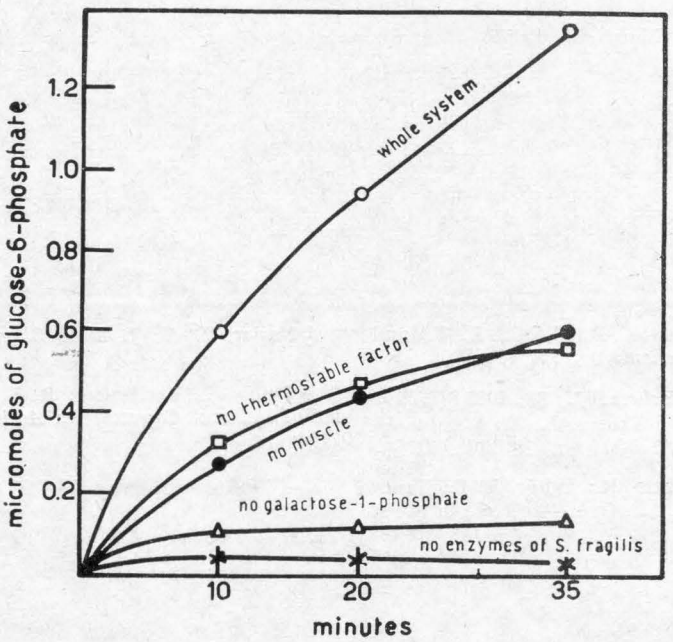

FIG. 1. The transformation of galactose-1-phosphate into glucose-6-phosphate. Whole system, 2 μM of galactose-6-phosphate, 1 μM of $MgSO_4$ 0.03 ml. of partially purified *S. fragilis* enzyme, 0.01 ml. of muscle extract containing phosphoglucomutase, and 0.05 ml. of purified thermostable factor from yeast; total volume, 2.3 ml. The glucose-6-phosphate is measured by its reducing power.[3]

$$\text{Galactose-1-phosphate} \xrightarrow[(a)]{} \text{glucose-1-phosphate} \xrightarrow[(b)]{} \text{glucose-6-phosphate}$$

In the absence of phosphoglucomutase, glucose-1-phosphate accumulates, as may be ascertained by destroying the *S. fragilis* enzyme by heating, adding phosphoglucomutase, and then measuring the glucose-6-phosphate formed.

The thermostable factor has been found to act in reaction *(a)*, and is diferent from glucose diphosphate, which acts in reaction *(b)*. This factor is present in mammalian liver and in commercial yeast. It is hoped that its identification will cast some light on the long sought mechanism of the inversion at C_4 in hexoses.

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Received for publication, March 24, 1949.

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ARCHIVES OF BIOCHEMISTRY
Vol. 24, Nº 1, November, 1949, p. 65

THE FORMATION OF GLUCOSE DIPHOSPHATE BY *ESCHERICHIA COLI*

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Received June 3, 1949

Introduction

Glucose-1,6-diphosphate[1] has been found to act as a coenzyme in the reaction:

Glucose-1-phosphate $\rightleftharpoons$ glucose-6-phosphate

when it is catalyzed by yeast or animal tissue phosphoglucomutase (2). During this latter investigation it was observed that glucose-1-phosphate solutions became contaminated with appreciable amounts of glucose diphosphate when stored for some time in the cold. From such solutions it was possible to isolate several microorganisms. Of these, *Escherichia coli* was found to be responsible for the formation of glucose diphosphate. Several strains of this organism were examined, as well as *Aerobacter aerogenes* and *Klebsiella pneumoniae*. The synthesis of glucose diphosphate occurred in the presence of any of these, whereas of glucose synthesis was obtained in preliminary tests with *Bacillus cereus, Bacillus alkaligenes, Sarcina conjunctivae, Serratia marcescens* and *Staphylococcus aureus*.

This paper represents the results of a study of the formation of glucose diphosphate and the fermentation of phosphoric esters by living *E. coli*. Extracts of *E. coli*, wich catalyzed the synthesis of glucose diphosphate, were prepared and purification of the active system was attempted. The mechanism of the reaction and the action of factors which influenced its rate were also studied.

[1] The structure of this substance has been confirmed by synthesis from silver phosphate and 1-bromo-2,3,4-triacetyl-6-diphenylphosphonoglucose by Repetto *et al.* (1).

Methods

Cultures

For small scale experiments, *E. coli* was cultivated on agar slants. After 24 hr. incubation, the bacterial growth was washed off, centrifuged and resuspended in water to a density corresponding to an extinction coefficient of E. = 0.8 for 1 cm. at 470 mμ. For larger scale experiments, the bacteria were harvested from peptone broth cultures after 20 hr. of incubation at 30ºC with aeration. The bacterial mass was separated by means of a continuous centrifuge and then stored in the frozen state until used. Extracts obtained after some days of storage were often more active than those obtained from fresh bacteria.

Substrates

Glucose-1-phosphate was prepared as described by Sumner and Somers (3), glucose-6-phosphate according to Colowick and Sutherland (4), and fructose diphosphate after Neuberg and Lustig (5). The preparation of glucose diphosphate has been previously described (2).

Analytical Methods

Glucose diphosphate was estimated by determination of its coenzymatic activity with a yeast phosphoglucomutase (2). Inorganic phosphate was determined by the procedure of Fiske and SubbaRow (6). The inorganic phosphate liberated during 7 min. of hydrolysis at 100ºC by 1 *N* acid is referred to as acid-labile phosphate, and that portion of the organic phosphate not hydrolyzed under similar conditions is referred to as acid-stable phosphate. Glucose was determined by the Somogyi (7) procedure using the Nelson reagent (8). Fructose was determined according to the directions of Roe (9).

Results

The Formation of Glucose Diphosphate by Living E. coli

When the bacteria were suspended in a glucose-1-phosphate solution, an accumulation of glucose diphosphate occurred which rea-

ched a maximum and then decreased. The time at which the maximum was obtained was variable and was found to depend on the amount of bacteria added and on the initial concentration of the substrate. A graphic representation of a typical experiment appears in Fig. 1.

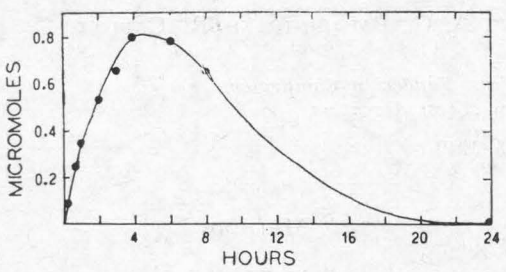

FIG. 1. — Glucose diphosphate formation by living E. Coli. Incubation at 37°C of 20 μM of glucose-1-phosphate, 1 ml. of M/15 phosphate buffer of pH 6.5 and 0.25 ml. of a suspension of E. coli. Total volume, 3.25 ml. Results in μM.

The formation of the diphosphate under aerobic and anaerobic conditions was aproximately the same. The maximum amount of diphosphate formed corresponded to the conversion of about 4 % of the initial amount of monophosphate added.

The formation of glucose diphosphate was measured with substrates other than glucose-1-phospate. No formation was detected from glucose (with or without added inorganic phosphate), glucose-6-phosphate, fructose diphosphate, saccharose, lactose, or maltose. The culture liquid remaining after centrifuging off the bacteria did not catalyze the formation of glucose diphosphate from the monophosphate.

The Fermentation of Phosphoric Esters

Manometric experiments showed that glucose-1-phosphate, glucose + inorganic phosphate, and glucose were rapidly fermented by *E. coli* (Table I) and also by *Aerobacter aerogenes* and *Klebsiella pneumoniae.*

Usually, glucose-1-phosphate was utilized at a rate slightly faster than the others. The rate of fermentation of glucose-6-phosphate was lower, while that of fructose diphosphate and glucose diphosphate was nearly undetectable.

TABLE I

Acid Formation by Living E. coli

CO_2 evolution measured in Warburg manometers containing: 0.025 *M* $NaHCO_3$, 4μ*M* of substrate and 0.4 ml. of a suspension of *E. coli.* Total volume: 2 ml. Temperature: 37°C. Results in μl. Gas: Nitrogen with 5 % CO_2.

Substrate	25 min.	50 min.
None	0	0
Glucose	30	60
Glucose + phosphate	33	67
Glucose-1-phosphate	37	75
Glucose-6-phosphate	17	35
Fructose diphosphate	7	8
Glucose diphosphate	8	10

These organisms differ from yeast, where glucose-1-phosphate is not fermented by intact cells. Evidently, there is a difference in permeability, since in *E. coli* glucose-1-phosphate is not hydrolyzed before entering the cells, for, if such were the case, glucose plus inorganic phosphate should be equivalent to glucose-1-phosphate in all respects. However, only the latter gives rise to glucose diphosphate.

Glucose Diphosphate Formation in Cell-Free Extracts

To study the mechanism of formation of glucose diphosphate from glucose-1-phosphate, it was deemed necessary to separate the enzyme system involved in this reaction. Crude extracts prepared from *E. coli,* acting on glucose-1-phosphate, were found to produce glucose diphosphate, together with considerable amounts of inorganic phosphate and reducing substances.

If the reaction is formulated as:

2 glucose-1-phosphate → glucose-1,6-diphosphate + glucose,

the molar ratio: glucose diphosphate/glucose should be 1. Actually, with the crude extracts, this ratio was found to be 0.01. The amount of inorganic phosphate liberated was roughly the same as that of reducing substances calculated as glucose, evidence which suggested that the main contaminating enzyme was a phosphatase. The increase in value of this

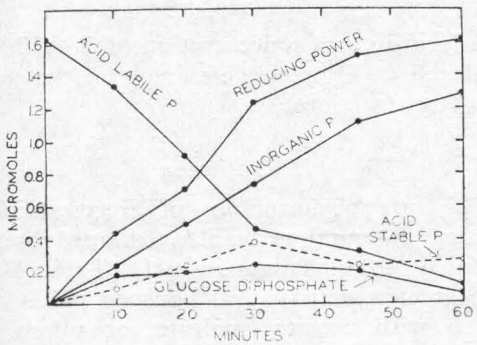

FIG. 2. Chemical changes produced by the enzyme 0.25 ml. samples containing 1.7 μM glucose-1-phosphate, 2 μM cysteine and 0.03 ml. of purified enzyme. Temperature, 37°C. Reducing power referred to a glucose standard.

ratio was, therefore, utilized as an index of the degree of purification of the diphosphate-forming enzyme. As can be observed in Fig. 2, the value of this ratio, with the same enzyme preparation, was found to depend on the length of incubation, and was approximately constant only during the first few minutes of the reaction.

Preparation of Cell-Free Extracts

Several procedures were attempted: Cytolysis with toluene, grinding with glass powder (10), and extracion of acetone-dried cells. The latter procedure was adopted, since it was found to yield more reproducible results. The dried bacteria, obtained as described by Harden (11) for the preparation of zymin, were extracted with 12 volumes of distilled water at 5°C. After 30 min., the suspension was centrifuged at 6000 r.p.m. The supernatant fluid was adjusted to pH 7, and solid $(NH_4)_2SO_4$ added to 0.5 saturation. The precipitate was removed by centrifuging and more solid $(NH_4)_2SO_4$ was added to the supernatant to bring the latter to 0.8 saturation wih respect to this salt. The resulting precipitate was separated and dissolved in water so that the volume was reduced to about 1/5 that of the original extract. This solution was then dialyzed 2-3 hr. in the cold. The dialyzed fluid contained about 50 % of the total activity of the original extract. Such preparations could be stored frozen for several weeks without deterioration.

The ratio: glucose diphosphate/glucose formed was 0.01 with the crude extract and 0.15 to 0.20 with the final dialyzed solution after activation by cysteine (see below). Repeated fractionation by $(NH_4)_2SO_4$ precipitation resulted in exttracts with a glucose diphosphate/glucose ratio of 0.35 to 0.50. Great losses of activity were encountered during this process of purification.

ACTIVATORS AND INHIBITORS

The Action of Cysteine and Magnesium

Dialysis of the extracts against cold water resulted in a gradual reduction of their activity so that, at the end of 6 or more hours of dialysis, the activity might have disappeared completely. The addition of magnesium ion was tested because it activates many of the enzymes which act on phosphoric esters. As shown in Table II, no increase in activity

TABLE II

The Action of Cysteine and Magnesium Ions on the Formation of Glucose Diphosphate

Incubation of: 2 μM glucose-1-phosphate with 0.03 ml. purified enzyme 10 min. at 37°C. Total volume, 0.25 ml.

	μM glucose diphosphate formed	Ratio: glucose diphosphate/glucose
Undialyzed enzyme	0.023	0.06
Dialyzed 4 hr.	0.015	0.02
Dialyzed + 1 μM Mg^{++}	0.015	0.02
Dialyzed + 1 μM cysteine	0.14	0.3
Dialyzed + 1 μM Mg^{++} + 1 μM cysteine	0.11	0.3

could be detected. However, when cysteine was added to the dialyzed extracts, the activity was increased 5- to 10-fold. On the other hand, the addition of cysteine had no effect on the liberation of reducing substances or inorganic phosphate. The optimum activating effect of cysteine was observed at a concentration of about 8×10^{-3} M.

The addition of boiled extracts of *E. coli* had no effect on the activity of the dialyzed enzyme.

The Action of Adenosine Phosphate

Glucose diphosphate has been found to be formed in muscle by transphosphorylation between glucose-1-phosphate and adenosinetriphosphate (12). The effect of the latter was investigated in the reaction as catalyzed by the extracts of *Escherichia coli*.

The effect of adenosinetriphosphate was tested at concentrations ranging from 10^{-5} to 10^{-2} M, and in no case could activation be detected. Concentrations higher than 10^{-3} produced an inhibition of the formation of

glucose diphosphate. Moreover, the maximum amount of adenine compounds present in the enzyme was calculated from the extinction at 260 mμ and the results showed that the amount of glucose diphosphate formed was at least 10 times larger than the maximum amount of adenine compounds present. No acid-labile phosphate could be detected.

It does not seem likely that any reaction which leads to phosphorylation of the adenosine compound takes place under these experimental conditions. In some experiments, the reaction was allowed to take place in War burg manometers at pH 7 in bicarbonate and a nitrogen-CO_2 gas phase. No acid formation was detectable and, therefore, reactions which would give rise to phosphorylation, such as the oxidation of glyceraldehyde or the formation of phosphopyruvic acid, could be excluded. These results appear to indicate that glucose diphosphate is not formed by transphosphorylation between ATP and glucose-1-phosphate.

Fluoride

Fluoride, at a concentration of 5.8×10^{-4} *M*, inhibited the phosphatase action to the extent of 75 % and retarded the rate of glucose diphosphate formation to about the same degree.

Phloridzin

Phloridzin at a concentration of 3×10^{-4} *M* produced no appreciable change on the course of the reactions.

pH Optimum

The rate of formation of glucose diphosphate was found to be affected only slightly by fairly large changes in pH (Table III). The greatest activity was between pH 5 and pH 6 with acetate, maleate, or phosphate buffers.

Chemical Changes Produced by the Enzyme Preparation

The course of the reaction involving the conversion of glucose-1-phosphate to glucose diphosphate by the purified enzyme was similar to that by intact cells. There was first an increase in glucose diphosphate followed by its gradual disappearance. It has not been possible to separate the formation from the destruction of the diphosphate by purification of the enzyme. Fig. 2 ilustrates the changes which occurred during the reaction. There was a gradual increase in inorganic phosphate and in the reducing power of the reaction mixture, coincident with a decrease in the acid-labile phosphate. At least 80 % of the reducing substances were not precipitated by the $ZnSO_4$-Ba $(OH)_2$ reagent (13), which precipitates the hexose phosphates.

TABLE III

pH Optimum

Incubation of 2 μ*M* of glucose-1-phosphate, 2 μ*M* of cysteine, 0.1 ml. of 0.1 *M* of acetate buffer and 0.03 ml. of purified enzyme. Total volume. 0.35 ml., 15 min. at 37°C. Results in μ*M*.

pH	3.8	4.4	4.7	5.3	5.9	6.2
Glucose diphosphate formed	0.08	0.15	0.17	0.23	0.19	0.15

These changes can be explained by the occurrence of the following reactions:

(a) 2 glucose-1-phosphate → glucose diphosphate + glucose,

(b) glucose-1-phosphate → glucose + inorganic phosphate,

(c) glucose-1-phosphate → glucose-6-phosphate.

Reaction (a) would be a transphosphorylation somewhat similar to the phosphoglucomutase reaction, which has been formulated (2) as follows:

(d) glucose-1-phosphate + glucose-1,6-diphosphate ⇌ glucose-1,6-diphosphate + glucose-6-phosphate.

Reaction (b) might occur directly or with (a) or (c) as intermediates. As to reaction (c), it is relatively slow as compared with the phosphatase (b) and, since large amounts of glucose diphosphate are formed, it was not possible to test whether it takes place by mechanism (d). Another possible mechanism for the formation of glucose-6-phosphate would be the removal of the phosphate at position one of glucose diphosphate by phosphatase.

The Effect of Glucose

Addition of glucose produced striking changes in the course of the reactions (Table IV). Whereas the formation of glucose diphosphate was not appreciably affected, there occurred a great increase in the acid-stable phosphate and a decrease in the liberation of inorganic phosphate. The amount of fructose was also increased.

TABLE IV

The Action of Glucose

10 μM glucose-1-phosphate +0.3 ml. enzyme solution + 10 μM cysteine. Total volume, 2 ml., 30 min. at 37°C. Results in μM. Values for fructose were corrected by subtracting the values found at $t = 0$.

Additions	Glucose diphosphate formed	P inorganic formed	Acid-stable P	Fructose
None	0.79	7.5	1.4	0.15
30 μM glucose	0.67	6.2	3.2	0.80
60 μM glucose	0.71	4.9	4.0	1.40

Sorbitol or ethanol did not produce an effect similar to glucose, whereas fructose was about half as effective at the same concentration.

The stable ester, which was formed in larger amounts in the presence of glucose, was presumably glucose-6-phosphate which was in equilibrium with fructose-6-phosphate. This explains the concomitant increase in the "fructose" content of the reaction mixture.

The action of glucose was similar to that described with liver enzymes (14), where it has been interpreted to be due the inhibition of a specific phosphatase acting on glucose-6-phosphate.

The partially purified enzyme of *E. coli* was found to liberate inorganic phosphate, not only from the glucose phosphates but also from the α- and β-galactose-1-phosphates, α- and β-glycerophosphates, and phenylphosphate. Of these, phenylphosphate was the most rapidly hydrolyzed.

Glucose was found to inhibit also the liberation of inorganic phosphate from α-galactose-1-phosphate.

DISCUSSION

The ready fermentability of glucose monophosphate by live *Escherichia coli* and related organisms reveals a difference with intact yeast cells, which do not measurably utilize phosphoric esters. Presumably, the cell membrane of *E. coli* is permeable to monophosphoric esters and, to a lesser extent, also to diphosphoric esters. Thus, during the fermentation of glucose-1-phosphate, the diphosphate is formed, passes to the medium, and is utilized at the end of the fermentation.

Glucose diphosphate has been found to be an intermediate in the utilization of glucose-1-phosphate, and this raises several problems, such as the mechanism of its formation and destruction and its role in the normal metabolism of *E. coli*

The formation of glucose diphosphate appeared to take place by a mechanism different from that in animal tissues or yeast, where it has been demonstrated that glucose-1-phosphate is transphosphorylated by adenosinetriphosphate (12). Definite proof of the mechanism of synthesis of glucose diphosphate by *E. coli* must be deferred until the specific enzyme system involved can be separated from interferings systems. However, evidence at hand can best be interpreted by assuming that the conversion of glucose-1-phosphate to glucose diphosphate involves a

transfer of phosphate from position-1 of glucose-1-phosphate to position-6 of another molecule of the same substance.

The utilization of glucose diphosphate will require further investigation. The partially purified preparation which catalyzed its synthesis appeared to break it down mainly by the action of a contaminating phosphatase. Some experiments designed to detect in crude extracts an enzyme similar to aldolase, but which would act on glucose disphophate, were not successful.

Summary

Glucose diphosphate was found to be formed by *Escherichia coli, Aerobacter aerogenes* and *Klebsiella pneumoniae* when incubated with glucose-1-phosphate. No formation was detected from other sugars or their derivatives. Living cells fermented glucose monophosphates and free glucose at about the same rate, and fructose disphosphate or glucose diphosphate hardly at all.

A partially purified enzyme was prepared which transformed glucose-1-phosphate into reducing substances and inorganic phosphate, with the transient formation of glucose diphosphate.

The formation of glucose diphosphate was activated by cysteine and inhibited by fluoride. Glucose decreased the rate of liberation of inorganic phosphate and increased the formation of acid-stable phosphoric esters.

It is postulated that glucose diphosphate is formed by transphosphorylation between two molecules of glucose-1-phosphate.

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ARCHIVES OF BIOCHEMISTRY
Vol. 22, p. 87, 1949

THE ISOLATION OF THE COENZYME OF PHOSPHOGLUCOMUTASE

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Received November 23, 1948; revised January 14, 1949

INTRODUCTION

Previous papers (1,2) reported the existence of a coenzyme for phosphoglucomutase and its probable identity with glucose-1,6-diphosphate. The isolation and properties of the substance and its role in animal tissues are now described.

The rate of the reaction: glucose-1-phosphate → glucose-6-phosphate is, under certain conditions, proportional to the concentration of coenzyme which can thus be easily estimated (Fig. 1).

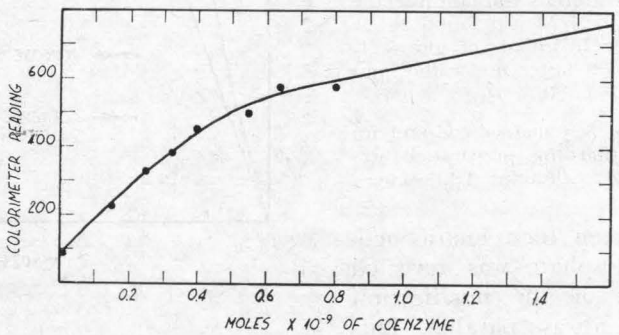

FIG. 1. Relationship between the amount of coenzyme and the activity of yeast phosphoglucomutase.

TABLE I

Results of the Puritzation of the Coenzyme

Step	Amount of extract	Micromoles of coenzyme	Relation P total/P acid-labile
1. Filtered yeast extract	1700 ml.	650 *a*	226 *b*
2. After Pb and H_2S	1300 ml.	680 *a*	130 *b*
3. Alkali, Mg++, Pb, H_2S	580 ml.	—	15 *b*
4a. First Ba salt	5.06 g.	545 *a* 680	10
4b. Fractionated Ba salt	1.85 g.	450	6.5
		F_1 50	2.2
5a. Acetone precipitation	—	F_2 128	2.1
		F_3 107	2.5
		F_4 28	3.8
5b. Final Ba salt ($F_1 + F_2$)	150 mg.	140	2.07

a Estimations by enzymatic method
b P total/μM of coenzyme.

During a search for the best starting material, and following the observation of Kendal and Stickland (3) that the phosphoglucomutase of rabbit muscle is activated by fructose diphosphate, this substance was tested. The sample used was found to contain considerable amounts of coenzyme. It was next observed that, during the incubation of yeast with sugar, phosphate and ether, as described by Neuberg and Lustig (4) for the preparation of fructose diphosphate, the coenzyme content increased enormously.

The separation of these two substances proved to be very difficult. For instance, a preparation which had been purified by barium, lead and alcohol fractionation, gave the same relation of coenzyme to fructose as the starting material. The only procedure by which the fructose ester could be removed was the destruction by heating at alkaline reaction. Large amounts of inorganic phosphate are then liberated, since the amount of coenzyme is only about 0.5 % of the other phosphoric esters. The removal of the inorganic phosphate with magnesia mixture often resulted in great losses by coprecipitation. This difficulty was finally overcome, and, after purification of the product by lead, barium, and acetone fractionation, a substance of about 70 % purity was obtained.

About half the phosphate present in these preparations is hydrolyzed by mild acid with loss of the coenzymatic activity. The hydrolysis constant for the first phosphate at 37ºC. in 0.25 *N* acid found to be 3.1 × 10-4 (Table II). The hydrolysis of glucose-1-phosphate is about four times faster under the same conditions ($K = 1.29 \times 10^{-3}$) (5).

The fact that the value of *K* remained constant up to 65 % hydrolysis shows that the preparation was not appreciably contaminated with other labile esters.

The connection between coenzymatic activity and acid-labile phosphate was revealed during the last stages of the purification where both values were always parallel. This parallelism was also evident during the acid hydrolysis (Table II).

Heating in 0.1 *N* acid at 100ºC. leads to complete liberation of the labile phosphate in 9-10 min. Fig. 2 shows the course of the hydrolysis compared with that of glucose-1-phosphate.

After it fas proved that the labile phosphate is related to the activity it became necessary to investigate the nature of the rest of the molecule.

The intact coenzyme gives a barium salt which is insoluble in water, but after hydrolysis most of the part bearing the second phosphate becomes water soluble. This indicates that the labile and stable phosphate belong to the same molecule.

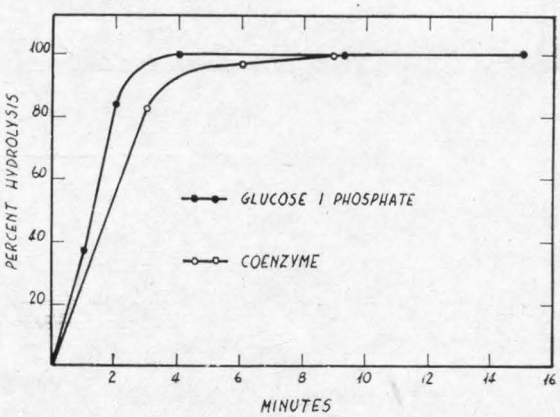

FIG. 2. — Hydrolysis of the labile phosphate of the coenzyme and of glucose1-phosphate in 0.1 *N* H_2SO_4 at 100ºC.

TABLE II

Hydrolysis of the Coenzyme in 0.25 N HCl at 37ºC.

$$K = \frac{1}{t_2 - t_1} \log_{10} \frac{100 - x_1}{100 - x_2}$$

Time	Liberation of P inorg. Per cent of acid-labile	$K \times 10^4$	Decrease in coenzymatic activity
min.			*per cent*
60	3.50	2.67	—
180	11.4	3.08	0
360	22.0	3.11	23
540	32.5	3.50	37
720	39.7	2.78	34
1440	64.5	3.25	77
		Mean: 3.06	

The estimation of aldose by titration with hypoiodite revealed that after mild acid hydrolysis one equivalent appeared per molecule of phosphate split off. None was reactive in the intact molecule.

These facts indicated that the second phosphate belonged to an aldose phosphate, and the enzymatic detection of glucose-6-phosphate was attempted. The procedure was based on the fact that muscle extracts contain enzymes which catalyze the equilibrum glucose-1- phosphate $\rightleftharpoons$ glucose-6-phosphate $\rightleftharpoons$ fructose-6-phosphate. Using aged and fairly dilute extracts, it is unlikely that other reactions will interfere. Moreover, since fructuose can be easily estimated, this affords a method for detecting any one of the 3 esters. The coenzyme before and after hydrolysis was incubated with the extract, and fructose was then estimated. As shown in Table III, the expected amount of fructose was formed from the hydrolysed coenzyme, and none from the nonhydrolyzed.

The correct identification of glucose-6-phosphate with relatively small amounts of material is a problem which has not been worked out satisfactorily. Two experiments were carried out in which polarimeter readings were taken before and after hydrolysis. The values obtained before hydrolysis gave $[\alpha]_D = +63^\circ$ to $+70^\circ$ in acid solution and calculated for an hexose diphosphoric acid. After hydrolysis, $[\alpha]_D = +30^\circ$ to $+32^\circ$ for an hexose monophosphoric acid. For glucose-6-phosphoric acid, the corresponding value is $+34.2^\circ$ to $+35.1^\circ$ (6,7).

The solution was then separated into water-soluble and insoluble barium salts. The soluble fraction gave $[\alpha]_D = +12^\circ$ to $+15^\circ$, calculated for the barium salt of an hexose monophosphate. The value given by Robison and King (6) for glucose-6-phosphate is $+16.6^\circ$.

TABLE III

Formation of Fructose Phosphate from the Hydrolyzed Coenzyme
(details in text)

Incubation for 20 min. at 37°C. of 0.1 ml. of enzyme solution plus additions. Total volume 0.5 ml.

Additions	Colorimeter readings
6.2 μM of coenzyme	12
6.2 μM of coenzyme hidrolyzed	128
2 μM glucose-1-phosphate	45
5 μM glucose-1-phosphate	98
Enzyme alone	8

Since these results were not considered conclusive, additional information was sought in the acid and alkaline hydrolysis and by preparing the osazone. The results were as follows:

	Soluble Ba salt from hydrolysis of coenzyme *per cent*	Glucose-6-phosphate *per cent*
Hydrolysis, 1 N acid at 100°C., 1 hr.	0—1	1.8 (6)
Hydrolysis, 1 N acid at 100°C., 2 hr.	4—5	3.5 (6)
Hydrolysis, 0.2 N alkali at 100°C., 3 min.	54	60 (10)

The osazone was undistinguishable microscopically from that of glucose-6-phosphate, and its melting point was 150-153°C. The value given by Robison and King [6] being 154°C.

If it is admitted that glucose-6-phosphate is formed by hydrolysis of the coenzyme, then in the intact substance the position of the labile phosphate can be fixed in 1 because of its facile hydrolysis with acid and because it masks the reducing group. The coenzyme would be, therefore, glucose-1,6-diphophate, and its high dextrorotation, which decreases on hydrolysis, indicates the α-anomer.

The presence of an aldose component in fructose diphophate preparations has been discussed by Neuberg, Lustig and Rothenberg [26]. Due to the small proportion of the glucose ester, it may have escaped detection by chemical methods or it may have been removed in the highly purified preparations used by Neuberg *et al.*

The synthesis of glucose diphosphate was attempted by treating 1,6-dibromotriacetylglucose with Ag_3PO_4, using the procedure of Cori, Colowick and Cori [5], which proved successful for the synthesis of glucose-1-phosphate. While an active preparation was obtained, thus adding more evidence to the proposed structure, the yield was so low, and purification so difficult, that the method was abandoned. The synthesis starting with glucose-1-phosphate and phosphorus oxychloride was unsuccessful.

All the facts which have been mentioned agree with the proposed structure of the coenzyme, but, since analytically pure preparations have not been obtained, other methods of synthesis are being investigated.

The structure of the coenzyme is important from the point of view of the mechanism of action of the enzyme. Meyerhof *et al.* [11] had foud that during the reaction there was no interchange between the hexose phosphates and radioactive inorganic phosphate. Schlamawitz and Greenberg [12] foud that labeled glucose did not interchange with the phosphorylated glucose, and postulated that a 1,6-glucose monophosphte was formed as an intermediary in the reaction.

A simple explanation for the mechanism, is that the enzyme would catalyse the transfer of the phosphate-1 of the coenzyme position 6 of glucose-1-phosphate. The reaction products would thus be glucose-6-phosphate and glucose-1,6-diphosphate. In the reaction the coenzyme would be regenerated at the expense of the substrate.

Another intriguing point has been settled. Gori, Colowick and Cori [13] had found that rabbit muscle phosphoglucomutase coul be electrodialyzed without losing activity. They could not reproduce the activation which Kendal and Stickland [3] had observed on adding fructose diphosphate preparations. Moreover, phosphoglucomutase has been purified by Schlamowitz and Greenberg [14], and crystallized by Najjar [15], and the necessity of a coenzyme was not noticed.

Since the coenzyme was found to activate yeast phosphoglucomutase but hardly at all that rabbit muscle, it seemed posible that the mechanism of action of the two enzymes might be different. However, it has been found that, if the muscle enzyme is tested in the presence of cysteine, as described by Najjar [15], then the addition of coenzyme produces a great increase in activity (Fig. 4, left).

If the muscle phosphoglucomutase is treated with acid in $(NH_4)_2SO_4$ solution as described by Warburg and Christian [16], for the reversible splitting of flavoproteins, then the activity without coenzyme becomes negligible (Fig. 4, right).

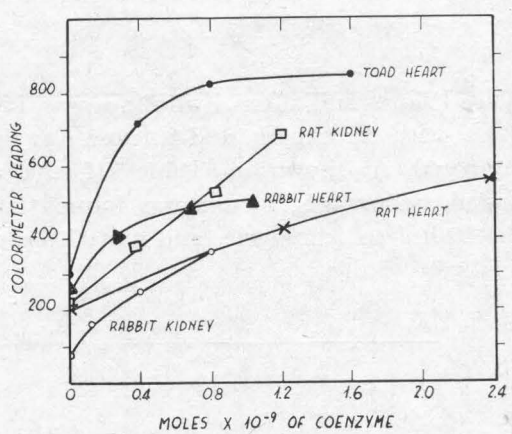

FIG. 3. — The activation of phosphoglucomutase from different organs by different amounts of coenzyme. Measurement by the copper reduction method. No cysteine used.

In extracts of heart and kidney the effect of the coenzyme can be observed even without the addition of cysteine (Fig. 3).

The coenzyme has been found in every animal tissue thus far investigated (Table IV), and it can be concluded that it is present wherever phosphoglucomutase is present.

EXPERIMENTAL

Enzymatic Estimation of the Coenzyme

Dried brewer's yeast was suspended in 3 vol. of water, incubated 2 hr. at 37°C., and then centrifuged. The supernatant was then diluted with 4 vol. of water. The activity was found to be unchanged on storage in the frozen state for several months. This crude extract was found to be activated by the coenzyme as much as dialyzed or $(NH_4)_2SO_4$-purified preparations.

The measurements were carried out in 12 × 100 mm. test tubes graduated to 7.5 ml. The reaction mixture contained 2 μM of sodium or potassium glucose-1-phosphate 1 μM $MgSO_4$, 0.02 ml. of enzyme solution and coenzyme solution. Total volume 0.3 ml. The reaction was started by adding the enzyme and, after 10 min. at 37°C., it was interrupted by addition of 1.5 ml. of Somogyi's sugar reagent (17).

The tubes were heated 10 min. in a boiling water bath, and then cooled. After addition of 1.5 ml. of Nelson's arsenomolybdic reagent (18) and water to complete the volume the color was measured with a Klett photocolorimeter fitted with filter 52. The values obtained with increasing amounts of coenzyme are shown in Fig. 1.

Since no buffer was used, all the samples were adjusted to pH 7.4-7.5. Unknown solutions were compared with a standard coenzyme, blanks without glucose phosphate were run at the same time. These blanks are necessary because crude solutions may reduce initially or may develop reducing power during incubation. For instance, samples containing saccharose give a high blank, due to the invertase present in the yeast extract. If blanks are high, it is convenient to purify partially by precipitation with lead acetate.

The most frequent cause of error is the presence of salts which inhibit the enzyme, as was observed by Cori, Colowick and Cori (13). Sometimes a precipitation with lead acetate is useful in this case also.

Substances with SH groups hardly affect the activity with the yeast preparations and, moreover, they can be destroyed by heating in alkaline solution.

Usually, 2 or 3 different amounts of the unknown, were compared with a curve obtained in the same series using 2 or 3 samples of the standard solution. The error amounted to about 10 %.

The Isolation of the Coenzyme

1. *Preparation of the Yeast Extract.* The technique of Neuberg and Lustig for preparing fructose diphosphate (4) was used with fresh baker's yeast, saccharose, and ether. The coenzyme content increased during incubation at 30°C. up to 24 hr., and slowly decreased thereafter:

Hours	0	2	5	24
Coenzyme content (μM/l.)	11	185	415	445

The substitution of saccharose for glucose or starch did not improve the yield.

After incubation, the proteins were coagulated by heating, and the mixture filtered through fluted paper.

2. *Precipitation with Lead Acetate.* To the filtrate of step 1,2 ml.-% of glacial acetic acid were added, and then excess of 25 % lead acetate. The suspension was filtered on Buchner funnels using "Celite super cell." The cake was suspended in water and decomposed with H_2S. After filtration and aeration, the liquid was made alkaline to phenolphthalein with NaOH.

Comments. The treatment with H_2S should be carried out rapidly, since the coenzyme is acid-labile. The omission of step 2 did not give good results.

3. *Destruction of Fructose Diphosphate.* The liquid from step 2 was treated with 0.05 vol. of 5 N NaOH, and then heated in a large boiling water bath. Half an hour after the thermometer had reached 90°C., the liquid was cooled. Acetic acid was added to pH 7, and then an excess of magnesium acetate. The mixture was left overnight in the icebox. The $Mg_3(PO_4)_2$ was filtered off and NH_4OH added until phenolphthalein gave a rose color. After filtration, the absence of inorganic phosphate in the filtrate was checked analytically. Excess lead acetate was then added. The suspension, which was slightly alkaline to litmus, was filtered, and the precipitate decomposed with H_2S, filtered and aerated.

Comments. During the alkaline treatment the liquid becomes dark brown, and nearly all the organic phosphate is hydrolyzed. The Seliwanoff reaction for fructose (19) becomes negative. The amount of alkali can be increased up to 1 N and the heating up to 3 hr. without appreciable destruction of coenzyme.

The elimination of the inorganic phosphate with magnesia mixture in the usual manner resulted sometimes in a loss of about 40 % of the coenzyme by coprecipitation. Excess NH_4OH should be avoided, since it interferes in the subsequent lead precipitation.

4. *Barium Fractionation.* Excess barium acetate and 0.2 vol. of alcohol were added, and the liquid was filtered using Celite super cel. The dry precipitate (4a, Table I) was suspended in 10 vol. of water, cooled in ice, and adjusted to pH 3.5 (bromo-phenol blue) with HCl. The precipitate was discarded, and the liquid was treated with 3 vol. of 96 % ehtyl alcohol. The acid barium salt was separated wtih hardened filter paper, dissolved in water, adjusted with

$Ba(OH)_2$ to pH 8, ond 0.2 vol. of alcohol was added. The precipitate was then centrifuged and dried with alcohol and ether (4b, Table I).

Comments. This fractionation has been described by McFarlane (20) for the purification of fructose diphosphate. In some cases, the procedure was repeated, and in others the acid barium salt was dried and treated directly as described in 5. The latter procedure is to be preferred.

After this step it can be considered that all the acid-labile phosphate corresponds to the coenzyme since all he fructose diphosphate has been destroyed and glucose-1 phosphate is separated as the barium soluble salt.

5. *Precipitation with Acetone.* The barium salt wa's suspended in water as a thick paste, cooled, and 5 *N* H_2SO_4 was added until thymol blue gave a rose color. After checking for the absence of barium ions, the suspension was filtered on a Buchner funnel through Celite and a small amount of Norit. The filtrate was treated with 10 vol. of acetone and centrifuged. The oily precipitate was dissolved in a small volume of water and stored in ice (Fraction 1). To the supernatant a drop of concentraded NH_4OH was added followed by centrifugation. Three or four fractions were collected in this manner and analysed separately for inorganic, acid, acid-labile and total phosphate (5a, Table I). The fractions which showed a relation total phosphate/acid-labile near 2 were mixed, acidified with acetic acid and treated with $BaCl_2$. The $BaSO_4$ was centrifuged off, and washed. The combined supernatant and washings were adjusted to pH 8 with filtered saturated $Ba(OH)_2$, 0.2 vol. alcohol added and centrifuged. The precipitate was washed with 20 % alcohol until the supernatant gave no reaction for chloride and then dried with alcohol-ether (5b). The yield and purification in different steps are shown in Table I. One of the barium salts obtained in this manner and dried *in vacuo* over $CaCl_2$ had an acid-labile phosphate content of 3,48 % and 8.5 % of total phosphate. For an anhydrous dibarium hexose disphosphate the theoretical total phosphate is: 10.15 %. The Seliwanoff reaction (19) for fructose gave values which were equal to those given by equivalent amounts of glucose. In some preparations, the values were slightly higher but could be lowered by a second treatment with alkali.

Acid Hydrolysis

The hydrolysis was carried out in 0.25 *N* HCl at 37° ± 0.1°C., and both the phosphate split off and the coenzymatic activity were determined. The preparation which had a P total/P acid-labile ratio of 2.4 and contained only about 3 % fructose [Roe (19) procedure] was freed from barium by adding the exact amount of H_2SO_4, after centrifuging it was diluted with one volume of 0.5 *N* HCl. It was then immersed in a thermostat, and the samples taken at intervals were pipetted into tubes containing sufficient amount of 0.3 *N* NaOH to neutralize the acid. Both inorganic and acidlabile phosphate were estimated by the method of Fiske and SubbaRow (21). The hydrolysis, which occurs during the development of color is negligible, and no correction was applied for it.

As a check on the whole procedure an experiment was run with glucose-1-phosphate, the value for *K* was found identical to the one given in the literature (5).

For analytical purposes the hydrolysis in 0.1 *N* H_2SO_4 at 100°C. was also studied. The results in Fig. 2 show that after 9-10 min. the phosphate liberated reaches a constant value.

Aldose Estimation

The method of Macleod and Robinson (22) was used, and a solution of the sodium salt of the coenzyme. Hydrolysis was carried out in 1 *N* HCl at 100°C. during 7 min. In one experiment, 0.71 ml. 0.02 *N* I_2 was used, and the phosphate split off in the same amount solution was 7.0 μM. Another experiment gave 0.75 ml iodine for 7.5 μM phosphate. That is the ratio aldose/labile phosphate was 0.9 and 1.0 respectively. No iodine was used by the nonhydrolyzed coenzyme. A check of the method with the same amounts of glucose gave 96 % of the theoretical value.

Enzymatic Identification of Glucose-6-Phosphate

An extract of rabbit muscle was prepared by mincing and extracting with 3 vol. of water. It was used after keeping several days in the ice box and the amount of enzyme necesary was determined in a preliminary experiment. One sample of the coenzyme was hydrolysed in 0.1 *N* acid 10 min. at 100°C., and then neutralized. Another sample was used intact. The tubes were incubated at 37°C., and then fructose was estimated as described by Roe (19). Results appear in Table III and show that the amount of fructose formed from 6.2 μM of the hydrolyzed coenzyme corresponds to about 6 5 μM of hexose phosphate.

Rotatory Power before and after Hydrolysis

A 2 dm. tube and sodium light were used in all the experiments.

Experiments I. A solution at pH 8 of the sodium salt of a preparation with a ratio total phosphate/labile phosphate = 2.02 was prepared. Concentration estimated by P content was 1.85 %, $\alpha = +1.72°$. Therefore, $[\alpha]D = +46.4°$, calculated for $C_6H_{10}O_6(Na_2PO_2)_3$.

The solution was made 1 *N* with HCl. Concentration = 1.18 g-% $\alpha = +1.50°$ Therefore, $[\alpha]D = +63.5°$ calculated for the free acid: $C_6H_{10}O_6(H_2PO_2)_3$.

The same solution was heated 10 min. at 100°C. and cooled: $\alpha = +0.58°$, which gives: $[\alpha]D = +32.2°$, calculated for: $C_6H_{11}O_6PO_3H_2$. The values in the literature for glucose-6-phosphoric acid are: $[\alpha]D = +35.1°$ (6) and $+34.2°$ (7).

The liquid was then neutralized, and excess barium acetate was added. The precipitate was centrifuged off and the supernatant precipitated with two volumes of ethyl alcohol. The precipitate was dried, redissolved in water and clarified by centrifugation $\alpha = +0.17°$, concentration = 0.586 g-% calculated from phosphate content. This gives $[\alpha]D = +15°$ for $C_3H_{11}O_6PO_3Ba$. The value is somewhat lower than that of barium glucose-6-phosphate. Robinson and King (6) give $[\alpha]_{5461} = +19{,}6°$; from this $[\alpha]D = +16°6$, ($[\alpha]D = [\alpha]_{5461}/1.18$) at 0.5 % concentration and $+18°$ at 8.4%. Other values given in the literature are: 17.9° (9), + 17.4 (8), + 16.6 (7).

Experiment II. A preparation of the barium salt with a ratio total phosphate/labile phosphate = 2.29 was dissolved in 0 07 *N* HCl Concentration calculated from the labile phosphate was 1.86 % $\alpha = + 2.66^{\circ}$. Therefore, $[\alpha]D = + 71.5^{\circ}$ for the free acid.

The liquid was made 0.2 *N* with HCl ond heated 10 min. at 100°C. Concentration = 1.32 %, and $\alpha = + 0.79^{\circ}$. This gives $[\alpha]D = + 30.0^{\circ}$ for an hexose monophosphoric acid.

The solution was then separated into water soluble and insoluble barium salts, as in Exp. I. Both were dried and studied. From 242 μM of starting material 183 of inorganic phosphate were recovered, 150 of barium soluble ester, and 51 of barium insoluble ester.

The barium soluble fraction gave $[\alpha]D = + 13^{\circ}$, calculated for a barium hexose monophosphate.

The barium insoluble fraction dissolved in 0.2 *N* HCl gave $\alpha = + 0.26^{\circ}$, and concentration was 0.0134 *M*.

Properties of the Product of Hydrolysis

The water-soluble barium fraction from the previous experiments was heated at 100°C. in 1 *N* H_2SO_4. The phosphate liberated was 0 in 1 hr., and 4 % in 2 hr. In another exp., 1 and 5 % respectively. For glucose-6-phosphate, Robison and King (6). give 1.8 and 3.5 %.

Alkaline hydrolysis was carried out in 0.2 *N* NaOH. Phosphate was estimated with higher acid concentration so as to decrease the interference silicate (23). Blanks run under identical conditions were substracted. Robison and MacFarlane (10) give a value of 60 % under the same conditions.

The osazone was prepared as described by McCready and Hassid (24); a copious precipitate was formed, which was compared with the osozone of a mixture of glucose-6-phosphate and fructose-6phosphate. By microscopic examination both samples were undistinguishable.

After recrystallization from alcohol-chloroform the melting point wa 150°-153°C. Robison and King (6) give 154°C.

Synthesis from 1,6-Dibromotriacetylglucose

4.5 g. of 1,6-dibromotriacetylglucose (25) were dissolved in 30 ml. of anhydrous benzene, and refluxed 2 hr. with 7 g. of Ag_3PO_4. In other experiments, longer times of heating were tried with no better results. After filtration the benzene was removed under reduced pressure. The solid was then dissolved in 40 ml. methanol plus 1.6 ml. of 5 *N* HCl. After 15 hr. at room temperature, the solution was neutralized, and excess barium acetate was added. After 2 hr., the precipitale was separated, washed with 20 % alcohol and dried. Obtained: 1 g. of a substance containing 0.66 of inorganic P, 0.85 of acid-labile and 0.29 of acid-stable phosphate (μM/mg.) The substance was active as coenzyme, 1 mg. having the same activity as 0.05 μM of the pure coenzyme. Yield calculated from the bromoacetyl compound 0.5 %. Four different preparations were carried out varying some details, but the yield was not improved.

Distribution of the Coenzyme in Animal Tissues

The organs from freshly killed animals were weighed, minced and suspended in 2 vol. of water. The proteins were removed by heating and centrifuging, the liquid was heated 20-30 min. at pH 9, and then adjusted to pH 7.4. The coenzyme was then estimated on suitable dilutions by the yeast phosphoglucomutase test. Results in Table IV.

TABLE IV

Coenzyme Content of Animal Tissues
μM/g.

Tissue	Rat	Rabbit	Toad
Liver	0.012-0.014	0.017	0.06
Kidney	0.009-0.0028	0.0025	—
Muscle	0.006-0.024	0.01-0.04	0.017
Brain	0.005-0.011	—	—
Heart	0.0028	0.05-0.09	0.0014

Action of the Coenzyme on the Phosphoglucomutase of Animal Tissues

Many experiments were carried out with the enzyme of animal tissues using essentially the same technique described for the estimation of the coenzyme. The extracts were obtained with 2 vol. of water and then adjusted to pH 7.5. The amount used was 0.005-0.02 ml.

As shown in Fig. 3, a considerable activation was obtained with the phosphoglucomutase of heart and kidney.

With muscle, the results were variable until use was made of the test described by Najjar (15), in which cysteine is used as an activator. The test was carried out as described by him with minor variations.

As shown in Fig. 4 (left), under these conditions,

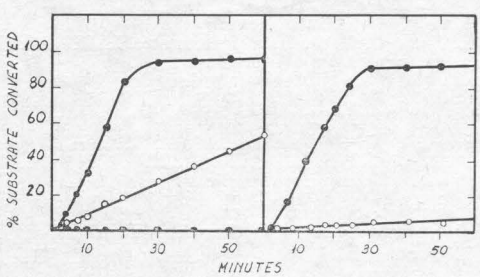

FIG. 4. — The action of the coenzyme on rabbit muscle phosphoglucomutase. Left: crude extract. Right: acid-treated extract. Upper curves: 0.1 ml. enzyme solution (see text), 2.3 μM glucose-1-phosphate, 5 μM cysteine, 1 u*M* $MgSO_4$ and 0.001 u*M* coenzyme, pH 7.5. Total volume, 0.5 ml. Temperature, 30°C. Lower curves (empty circles): the same without coenzyme. Lowest curve on left figure (black squares) same as upper but without cysteine.

the addition of coenzyme increases the rate of reaction about 300 % as compared with cysteine alone.

Still more demonstrative results were obtained when the method of Warburg and Christian (16) was applied. The preparations obtained in this manner showed practically no activity in the absence of coenzyme (Fig. 4, right). The procedure was as follows. A rabbit muscle extract was obtained by mincing and extracting with 2 vol. of ice cold water. After half an hour it was strained through muslin. For the experiment shown in Fig. 4 (left), this extract was diluted 100 times with cold water immediately before use. For the acid splitting: to 2 ml. of this extract plus 0.7 ml. of saturated $(NH_4)_2SO_4$, 3 ml. of 0.1 *N* HCl were added with stirring with the pipette submerged in the liquid. These operations, as well as the centrifugation, were carried out at 0ºC. The precipitate was then dissolved in 2 ml. of water and neutralized in the cold. After centrifuging off denatured protein, the preparation was ready for use and could be stored several days in the frozen state without loss of activity. In the experiment shown in Fig. 4 (right), this solution was diluted with 1 vol. of cold water.

Summary

Methods for the estimation and isolation of the coenzyme of phosphoglucomutase are described. The substance contains two phosphate groups. The first phosphate can be hydrolyzed by mild acid with the simultaneous liberation of an aldose group (hypoiodite titration).

The hydrolysis constant for the first phosphate in 0.25 *N* acid at 37ºC. was found to be 3.1×10^{-4}.

The coenzyme preparations gave a specific rotation $[\alpha]_D = +63^\circ$ to $+70^\circ$ calculated for an hexose diphosphoric ester. On hydrolysis the dextrorotation decreased to values comparable to those of glucose-6-phosphate. Other properties of the substance remaining after removing the first phosphate, such as behavior toward enzymes, acid and alkaline treatment, and the formation of an osazone, agreed with the properties of glucose-6-phosphate.

An active preparation was obtained by treating 1,6-dibromotriacetylglucose with Ag_3PO_4.

The structure of the coenzyme is, therefore, postulated as α-1,6-glucose diphosphate.

The reversible splitting of the phosphoglucomutase from rabbit muscle is described, as well as the distribuiton of the coenzyme in some animal tissues.

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ACTA PHYSIOLOGICA LATINOAMERICANA
Vol. 1, p. 57, 1950

LIVER URIDINE PHOSPHORYLASE

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Some animal tissues contain enzymes which split off the base from nucleosides. Klein [1] studied their action on purine and pyrimidine nucleosides and called them nucleosidases. He observed that their activity disappears after dialysis and reappears in the presence of phosphate or arseniate.

The mechanism of action one of these enzymes was elucidated by Kalckar [2]. In the course of studies on the estimation of nucleosides [3] he rediscovered the necessity of phosphate and was able to prove that ribose-1-phosphate was formed in the reaction. The latter substance is very acid-labile, so that the phosphate estimations had to be carried out with the method of Lowry and López [4] which avoids the use of strong acid.

Kalckar found that with a rat liver enzyme and inosine (hypoxanthine riboside) or guanosine as substrates, the following reversible reactions took place:

Hypoxanthine riboside + phosphate $\rightleftarrows$ hypoxanthine + ribose-1-phosphate Guanosine + phosphate $\rightleftarrows$ guanine + ribose-1-phosphate.

Due to the similarity of the reaction with that catalyzed by polysaccharide phosphorylase the enzyme was named nucleoside phosphorylase. The ribose-1-phosphate formed appears to be the furanoside since it was found that synthetic ribopyranose-1-phosphate was not used in the reaction going from right to left, that is in the synthesis of nucleosides.

Kalckar was unable to detect any action of the enzyme on adenosine, xanthosine or pyrimidine ribosides. However, the experiments of Schlenk and Waldvogel [5] show that ribose-phosphate is formed also from adenosine.

Similar reactions have been found to occur with desoxyribose-nucleosides. Manson and Lampen [6] obtained an enzyme from thymus which catalyzed the formation of desoxyribose-phosphate from hypoxanthine desoxyriboside and inorganic phosphate. The ester formed was beliefed to be desoxyribose-5-phosphate. They also detected a similar enzyme in bone marrow and kidney which acts on desoxypyrimidine-nucleosides, specially thymidine [7]. Similar results have been obtained by Wajzer [8].

The formation of desoxyribose-1-phosphate from guanine-desoxyriboside was detected by Friedkin, Kalckar an Hoff-Jorgensen [9]. The reaction was found to be reversible and the nucleoside could be synthesided from hypoxanthine and desoxyribose-1-phosphate [2] The latter substance was found to be even more acid-labile than ribose-1-phosphate.

Crude extracts of liver and other tissues catalyze the formation of ribose-5-phosphate from the 1-isomer. This reaction is similar to the transformation of the glucose-1-phosphate into the 6-phosphate which requires glucose 1,6-diphosphate as coenzyne [10]. It is therefore likely that the coenzyme of phosphoribomutase is ribose-1,5- disphosphate [11]. The existence of a similar enzyme for the desoxyribose-phosphate has been postulated by Manson and Lampen [6]

Schlenk and Waldvogel [5] have observed that when guanosine or adenosine is incubated with liver extracts and phosphate, the ribose disappears and is replaced by an acid stable phosphoric ester. They were able to prove [12] that about half the ribose-phosphate was transformed into hexose-6-phosphate. The same transformation ocurred starting with ribose-5-phosphate but not with free ribose or ribose-3-phosphate.

A similar observation had been made by Dische [13] several years before. In human red blood cells the ribose of adenosine disappeared. He suggested that the pentose was transformed into triose- phosphate and a two carbon compound. Using a bacterial enzyme Racker [14] detected the formation of triose-phosphate from ribose-5-phosphate. With muscle aldolase he observed a condensation of triose-phosphate with glycolic aldeyde to a pentose-phosphate. However, the product was not ribose-phosphate nor was there any evidence of its transformation into this substance.

The synthesis of inosinic acid has been reported by Wajzer and Baron [15-16] by incubation of inosine, ribose-3-phosphate and a liver enzyme. The inosinic acid was estimated by its activating action of polysaccharide phosphorylase.

Interest in the metabolism of uridine was aroused in this laboratory as a consequence of the isolation of uridine-diphosphate-glucose [17] which acts as a coenzyme in the galactose-1-phosphate → glucose-1-phosphate transformation. It was found that rat liver contains an enzyme which removes uracil from ribose, which is activated by phosphate and which leads to the formation of ribose-phosphate. The enzyme therefore catalyzes the phosphorolysis of uridine. In a note Paege and Schlenk [18] have recently reported the presence of a similar enzyme in bacteria.

Experimental

Preparation of the enzyme. — Rats were killed and the liver was immediately homogeneized in two volumes of water. After standing two or three hours in the ice-box, the homogenate was centrifuged at 3.000 r.p.m. The supernatant was then dialyzed against distilled water for 15 to 20 hours. Dialysis for a longer time did not inactivate the enzyme. On storage for several days at −5°C inactive protein precipitated and no appreciable loss of activity was observed. In some experiments the extract of acetone dried liver as described by Schlenk and Waldvogel [5] was used, and in others the extract prepared according to Kalckar [3] taking the fraction which precipitates between 0.4 and 0.6 saturated ammonium sulphate followed by dialysis.

TABLE I

Action of the liver extract on different substrates. Results in micromoles of ribose-5-phosphate

SUBSTRATE	Hours of incubation			
	1	2	3	5
Uridine	0.14	0.25	0.29	0.29
Uridine-3'-phosphate	0.12	0.17	0.21	0.24
Uridine-5'-phosphate	—	—	0.29	—
Cytosine	0	0	0	—
Cytosine-3'-phosphate	0	0	0	—

TABLE II

The action of phosphate and magnesium. Procedure as described in text. Substrate: uridine. Results in micromoles of ribose-5-phosphate

Hours of incubation	No phosphate No magnesium	No phosphate With magnesium	With phosphate With magnesium
1	0	0.02	0.15
2	0.03	0.02	0.16

TABLE III

The action of magnesium ion concentration. Procedure as described in text. Incubation during 2 hours at 37°C. Results in micromoles of ribose-5-phosphate

Molar concentration of Mg	0	.0013	.0025	.005	.01	0.02	0.04
Uridine	.09	—	0.12	0.15	0.20	0.22	0.22
Uridine-3'-phosphate	.04	0.07	0.10	0.11	0.15	0.18	—

TABLE IV

pH optimum. Results in micromoles of ribose-5-phosphate

SUBSTRATE	*pH*			
	6.6	7.0	7.4	8.0
Uridine	0.06	0.08	0.18	0.17
Urdiylic acid.	0.11	0.12	0.13	0.11

Substrates. — Uridine, uridine-3'-phosphate, cytosine and cytosine-3'-phosphate were prepared according to Loring *et al* [19] or were commercial samples (Schwarz). Uridine-5'-phosphate was prepared from uridine-diphosphate-glucose [17].

Estimation of the enzyme. — The ribose of pyrimidin-ribosides gives no colour with the methods used for pentose estimation. Therefore, the liberation of ribose or ribose-phosphate can be easily detected.

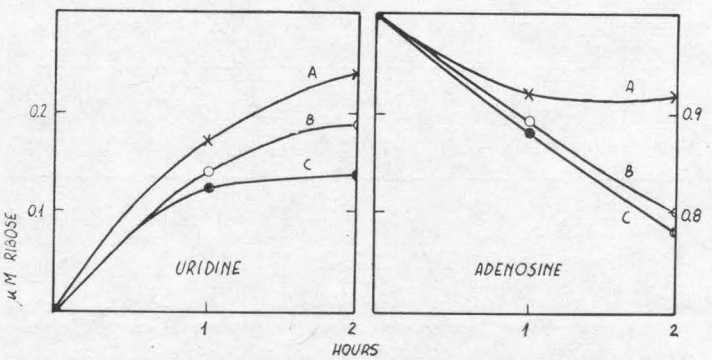

FIGURE 1. — The action of different enzyme preparations on uridine and adenosine.

x Liver extract dialyzed 20 hours.

• Fraction precipitating between 0.4 — 0.6 saturation with ammonium sulphate following the procedure of Kalckar (3).

o Extract of acetone dried liver according to Schlenk and Waldvogel (5).

The enzymatic system was made up as follows: 1 micromole of substrate, plus 0.1 ml of 0.06 M phosphate buffer of pH 7.4 plus 0.1 ml of 0.08 M magnesium chloride and 0.1 ml of enzyme solution. Total volume, 0.3 to 0.4 ml. Incubation was carried out at 37°C in the presence of toluene.

The reaction was interrupted at the desired time by addition of one volume of 2 N HCl; after centrifugation an aliquot of the supernatant, usually half, was taken. Water was then added to make 3 ml and the ribose was estimated following the method described by Mejbaum [20], heating 30 minutes. In some cases the coloured compound was extracted with amyl alcohol. Ribose or xylose were used as standards and the colorimetric values were calculated as ribose-5-phosphate following the indications of Albaum and Umbreit [21].

Results

Action on different substrates. — As shown in table I, the liberation of ribose could be detected with uridine or its phosphates, but not with cytosine. With the latter the addition of heated liver extracts was tested with negative results. The uridine-phosphates are probably transformed into uridine of phosphatase action.

Accurate results of the activity of uridine-phosphorylase were not obtained because the system contains enzymes which catalyze the disappearance of ribose-phosphate. This can be observed in fig. 1. When adenosine is the substrate the ribose moiety is directly estimated with Mejbaum's procedure. By incubation with the enzyme mixture the values decrease due to the further transformation of the ribose-phosphate liberated by the phosphorylase. With uridine the ribose moiety is not estimated until it is transformed into ribose-phosphate. The accumulation of ribose-phosphate from uridine depends on the relative activity of two reactions: the uridine-phosphorylase which forms ribose-phosphate and the other enzymes which destroy it. The results using the enzyme preparations of Kalckar and Schlenk and Waldvogel are also shown in fig. 1.

Conditions for maximum activity. — Results appearing in table II show that practically no enzymatic activity was detected in the absence of either phosphate or magnesium ions. The effect of the latter was more manifest after a thorough dialysis. The optimum concentration of magnesium is 0.02 to 0.04 M or higher (table III).

TABLE V

The formation of ribose esters. Procedure described in text. Amount doubled. Substrate: uridine. Ribose esters represent the part precipitated by the Somogyi deproteinizing procedure.

Hours of incubation	Free ribose μ moles	Ribose esters μ moles
1	0.04	0.30
2	0.04	0.50
3	0.06	0.53

TABLE VI

Liberation of uracil. Substrate: 2 micromoles of uridine. Uracil estimated by absorbancy at 290 mμ in 0.01 NaOH after deproteinizing with trichloracetic acid.

Hours of incubation	Ribose esters formed	Uracil liberated
2	0.40	0.46
3	0.46	0.54

The pH optimum was about 7.4 both when the substrate was uridine or uridylic acid (table IV).

The formation of ribose ester. – It is known that carbohydrate phosphates are precipitated in the deproteinization procedure described by Somogyi [22]. Use of this fact was made in order to find out whether the ribose liberated from uridine was free or esterified. The ribose left in the supernatant after zinc sulphate barium hydroxide treatment was substracted from the amount found directly. This value was considered to represent esterified ribose.

As shown in table V, practically all the ribose liberated by the enzyme is in the bound form.

The liberation of uracil. – The absorption spectra of uracil and uridine published by Ploeser and Loring [23] show that in 0.01 N sodium hydroxide the molar absorbency index (molar extinction coefficient) at 290 mμ for uracil is 5 500 and practically zero for uridine. It is thus possible to estimate these two substances in a mixture. As shown in table VI, the amount of uracil liberated is slightly higher than the amount of ribose esters formed.

Discussion

The reactions catalyzed by the enzyme mixture can be formulated as follows:

I Uridine + phosphate $\rightleftarrows$ uracil + ribose-1-phosphate.

II Ribose-1-phosphate $\rightleftarrows$ ribose-5-phosphate.

III Ribose-5-phosphate $\rightleftarrows$ unidentified products.

That reaction I takes place is proved by the liberation of uracil, by the indispensability of phosphate for the reaction and by the formation of ribose ester. Reactions II and III also take place with the extract used because starting with adenosine there occurs a disappearance of ribose, the mechanism of which has been studied by other workers.

The necessity of magnesium ions has not been mentioned in the work of Kalckar on guanosine-phosphorylase. It is likely that magnesium is necessary for reaction II and not for the phosphorylase (reaction I). The activation observed would be due to the fact that reaction I is reversible and would not take place if the ribose-1-phosphate is not removed from the reaction mixture by the phosphoribomutase (reaction II).

The activity of nucleoside phosphorylase which was found by Kalckar to be limited to the guanine and hypoxanthine ribosides, appears to be more general. Thus activity on adenosine was found by Schlenk and coworkers and activity on uridine is described in this paper. It is likely that the discrepancies are due to the methods of estimation and on the position of the equilibrium in these reactions.

Another point which remains to be settled is whether there is only one phosphorylase which acts on several ribosides or whether each nucleoside needs a specific enzyme.

Summary

Rat liver extracts were found to contain an enzyme which in the presence of inorganic phosphate transforms uridine into uracil and ribose phosphate. The process was found to be accelerated by magnesium ions.

Uridylic acid was also transformed whereas cytosine and cytidylic acid were not.

Resumen

En el extracto de hígado de rata se demostró la presencia de una fosforilasa que actúa sobre la uridina y los ácidos uridílicos originando, en presencia de fosfato y magnesio, uracilo y ésteres de ribosa.

La citosina y el ácido citidílico no son atacados.

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NATURE
Vol. 165, p. 191, February 4, 1950

URIDINE DIPHOSPHATE GLUCOSE: THE COENZYME OF THE GALACTOSE-GLUCOSE PHOSPHATE ISOMERIZATION

A PREVIOUS report [1] dealt with the enzymatic conversion of galactose-1-phosphate into glucose-1-phosphate and mentioned that a thermostable factor is necessary for the reaction. This factor has now been obtained practically pure, and for reasons given below will be referred to as uridine diphosphate glucose.

The preparation of uridine diphosphate was effected by extraction of bakers' yeast with one volume of ethanol, followed by fractional precipitation with mercuric acetate, adsorption on charcoal, elution with ethanol and treatment with a cation-exchange resin.

During the last stages of purification, it was observed that the activity ran parallel with a substance showing absorption at 260 mμ. The same parallelism was observed during paper chromatography with 77 per cent ethanol. The absorption spectrum of the substance is identical with that of uridine, and shows the same changes in acid and alkali, and after treatment with bromine. For each uridine calculated from its absorption coefficient [2], the substance contains two phosphates and two nitrogens.

On mild acid hydrolysis, uridine diphosphate loses its coenzymatic activity and yields a molecule of a reducing sugar which was identified as glucose by selective fermentation, paper chromatography, and the carbazole reaction [3] after removing interfering substances with ion exchange resins. The rate of glucose liberation in 0.01 *N* acid at 100°C. is higher than that of glucose-1-phosphate (half-life-time $t_{1/2}$ = 0.8 and 3.8 min. respectively).

Intact uridine diphosphate is non-reducing, and, together with the liberation of glucose, there occurs a change in the titration curve: it has two primary phosphoric acid groups and no secondary, while removal of the glucose unmasks a secondary acid group. This indicates that the glucose must be combined at position 1 with a phosphate group. Further evidence on the linkage of glucose to phosphate was obtained by treatment of uridine diphosphate with a suspension of washed kidney particles. A substance was liberated which behaved like glucose-1-phosphate under the action of phosphoglucomutase. Oxidation of uridine diphosphate with periodate gave one molecule of formic acid and no formaldehyde, as would be expected from a glucopyranosyl residue.

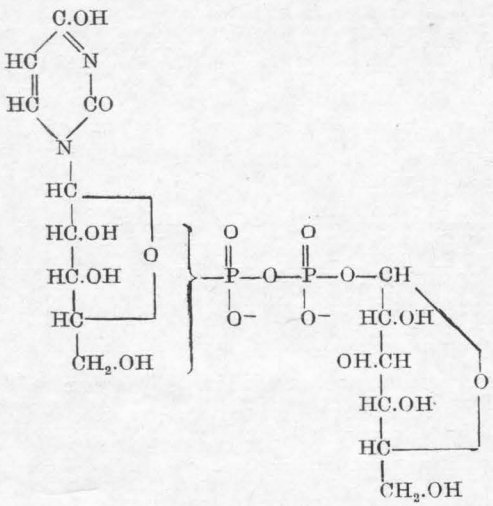
The substance remaining after removal of the glucose contains two phosphate groups, one of which is 90 per cent hydrolysed in *N* acid at 100°C. in 15 min. After hydrolysing this labile phosphate, the reaction mixture shows two primary and two secondary acid groups. The second phosphate group is very stable, and its rate of hydrolysis was found to be nearly equal to that given by Gulland and Smith [4] for uridine-2-phosphate. However, uridine-5-phosphate cannot as yet be excluded.

The substance remaining after removal of the two phosphate groups was identified as uridine by paper chromatography. The pentose could be estimated by the orcinol reaction after bromination [5]. Further hydrolysis in 6 *N* hydrochloric acid for 2 hr. at 120°C. gave uracil as judged by chromatography and by its spectrum at various *p*H.

On the basis of this evidence, uridine diphosphate can be tentatively assigned the accompanying formula, which shows a pyrophosphate linkage and explains the facts that it has only two primary acid groups, and that one secondary acid group appears on removal of the glucose residue and another on hydrolysis of the labile phosphate. Molecular weight calculated from the dry weight and uridine content gave a value of 630, which is not too far from the theoretical value of 566.

One point is not yet clear; that is the action of alkali. Treatment with 0.1 *N* alkali at

100°C. destroys the activity in a few minutes. No phosphate is liberated; but there occurs a stabilization of the glucosidic linkage so that the glucose now undergoes hydrolysis at the same rate as the first phosphate group. Presumably alkali brings about a rearrangement of the molecule.



The mechanism by which uridine diphosphate accelerates the conversion of galactose into glucose will require further investigation. The fact that glucose phosphate can be recognized as a part of the coenzyme suggests that this portion may exchange with the substrate during the reaction cycle.

An interesting fact is the similarity of uridine diphosphate with the compound which Park and Johnson [6] have found to accumulate in *Staphylococcus aureus* grown in the presence of penicillin. It is not possible from their data to conclude that it is the same compound; but the resemblance is striking.

A full account of this investigation will be published elsewere.

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 33, Nº 2, September 1951, p. 186

THE ENZYMATIC TRANSFORMATION OF URIDINE DIPHOSPHATE GLUCOSE INTO A GALACTOSE DERIVATIVE

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Received May 14, 1951

INTRODUCTION

A previous paper[1] reported the isolation of a substance acting as coenzyme in the transformation of galactose phosphate into glucose phosphate. The structure of this coenzyme (UDPG) [1] can be described as a glucose 1-phosphate molecule attached to uridine 5'-phosphate forming a pyrophosphate link.

In view of the fact that hexose phosphate is the substrate and also a part of the coenzyme, investigations were carried out in order to detect possible changes brought about by the enzyme on the sugar moiety of the coenzyme. The enzyme used was an extract of *Saccharomyces fragilis,* which can transform galactose 1-phosphate into glucose 1-phosphate in the presence of UDPG. It was found that when UDPG alone is incubated with this enzyme preparation a part of the glucose is transformed into galactose. As shown in Fig. 1, the reaction reaches an equilibrium in which about 25 % of the sugar is galactose and the rest glucose. Detection of galactose was carried out by paper chromatography with the pyridine-ethyl acetate solvent described by Jermyn and Isherwood[2]. Considerable experience with this procedure has shown that galactose can be distinguished from 20 other sugars or related compounds.

Other procedures such as selective fermentation or colored reactions while giving approximately the expected results were not convincing.

After it was found that a galactose-containing compound was formed by enzyme action on UDPG, information was sought on the identity of this compound. The most obvious possibilities were UDPGa, gal-1-P, or free galactose. Three types of experiments were carried out in order to settle this point.

(*a*) The nucleotides can be precipitated with mercuric salts leaving the esters and free sugars in the supernatant. The galactose-containing compound was found to precipitate with mercury like UDPG.

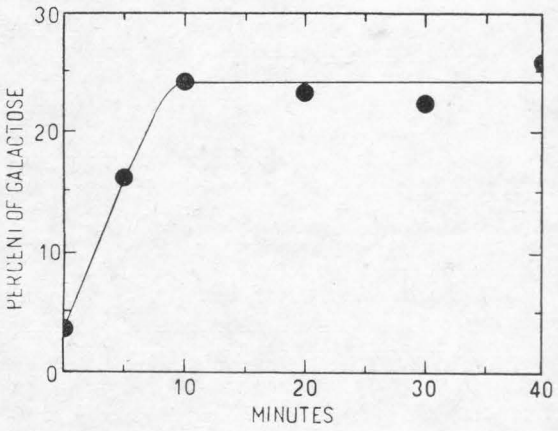

FIG. 1. — Formation of galactose by incubation of UDPG with *S. fragilis* extract. Incubation at 37º of 2 μmoles of UDPG with 0.04 ml. of dialyzed *S. fragilis* extract. After incubation 0.02 ml. of 2 *N* H_2SO_4 was added, the tubes were heated 15 min. at 100º and cooled. Three-tenths ml. of 0.3 *M* barium hydroxide was added and then 0.1 ml. of 5 % zinc sulfate. After centrifuging, the samples were evaporated and deposited on filter paper for chromatography (see text). Ordinates represent the per cent of galactose in total hexose.

(*b*) UDPG can be separated from the esters and free sugar by chromatography with ethanol-acetate at pH 3.8. Here the galactose compound was found in the UDPG zone.

1 The following abbvreviations are used: UDPG = uridine diphosphate glucose; UDPGa = uridine diphosphate galactose; glu-1-P = glucose 1-phosphate; gal-1-P = galactose 1-phosphate; UMP = uridine 5'-phosphate.

(c) It has been found [3] that when UDPG is chromatographed with ethanol-ammonia the compound breaks down to uridylic acid and a compound containing one glucose molecule and one phosphate which is doubly esterified. This compound will be referred to as the cyclic phosphate. This compound migrates in the solvent much faster than any of the known hexose monophosphates and slightly slower than free hexoses. In this type of chromatography it was found that the cyclic phosphate zone contained both glucose and galactose.

Therefore, all the experiments agree in that incubation of UDPG with the *S. fragilis* extract gives rise to a new compound in which the glucose is replaced by galactose. This compound may be referred to as UDPGa.

In order to correlate this enzymatic change with the catalytic role of UDPG in the transformation of galactose 1-phosphate into glucose 1-phosphate this reaction can be separated into two steps:

$$\text{UDPG} + \text{gal-1-P} \rightleftharpoons \text{UDPGa} + \text{glu-1-P}, \quad (1)$$

$$\text{UDPGa} \rightleftharpoons \text{UDPG}. \quad (2)$$

Reaction 1, which is hypothetical, is a transfer of UMP from glucose phosphate to galactose phosphate, while reaction 2 is proved to occur by the experiments reported in this paper.

As to the mechanism of reaction 2, one possibility would be an aldolase type of splitting between C-3 and C-4 of the hexose followed by recombination. This would necessitate the formation of free triose. Experiments designed to detect triose in the reaction mixture either with or without a trapping agent such as cyanide have been negative.

In connection with the cyclic phosphate formed by the action of ammonia on UDPG it may be mentioned that Forrest and Todd [4] have independently found that flavine adenine dinucleotide suffers a similar degradation which leads to the formation of a ribityl flavine monophosphate, which is doubly esterified at positions 4 and 5. The degradation product of UDPG is nonreducing, and therefore one point of attachment of the phosphate is at position 1. The other point of attachment had not been established. For an α-glucose derivative it might conceivably be either 1,2 or 1,4. Since UDPGa also gives rise to the diester and on steric grounds it would be highly improbable to have a 1,4-galactose compound, it seems reasonable to suppose that the substances in question are esterified at positions 1 and 2.

Experimental

The substrates and *S. fragilis* extract were prepared as described previously (1). For most experiments the enzyme preparation was dialyzed 6 hr. in the cold. The separation of galactose from glucose was carried out by descending paper chromatography using a pyridine-ethyl acetate-water (5:10:6) solvent (2) equilibrated at 30° before separation of the two phases.

The samples were deposited as a band on 2 cm. wide Whatman No. 1 filter-paper strips which had a blotting paper pad stapled on the end (5). Xylose was added in all the experiments as an internal standard. After running for 16-20 hr. the papers were dried, immersed in butanol-aniline-phthalate reagent (6) and the color was developed by heating to 105° for 5 min. With this reagent free hexoses give a brown color immediately; the 1-esters, UDPG, and the cyclic phosphates give faint colors and only after prolonged heating. For some experiments (Fig. 1), the spots were cut out, extracted overnight with 3.5 ml. of 0.7 *N* HCl in 80 % ethanol at room temperature and the optical density was measured with a Beckman spectrophotometer at 400 mμ. If appropiate glucose and galactose standards were run at the same time the results were satisfacory from the quantitative point of view.

(a) *Precipitation of the Galactose Compound with Mercuric Acetate*

Six micromoles of UDPG in 0.2 ml. of water was incubated with 0.05 ml. of *S. fragilis* extract for 15 min. at 37°. The control contained the same components but the enzymatic reaction was stopped at 0-time, and 2 μmoles of galactose 1-phosphate and of galactose were added. These addition were made in order to check the separation of UDPG from hexose esters and free sugars. After adding alcohol to 50 % concentration the samples were made acid to Congo red paper with nitric acid, and 0.1 ml. of 20 % mercuric acetate was added. The precipitate was dissolved in 1 ml. of 0.4 *M* ammonium acetate, and alcohol and nitric acid were added as before followed by 1 drop of mercuric acetate. After repeating the procedure two times more, the precipitate was suspended in water, decomposed with H_2S, and heated at pH 2 at 100° for 15 mins. The solution was then neutralized with barium hydroxide and centrifuged. The supernatant was then concentrated and chromatographed. Glucose and galactose were found to be present in the incubated sample while the control contained only glucose.

(b) *Chromatography with Acid-Ethanol*

One ml. of UDPG solution containing 15 μmoles

was mixed with 0.2 ml. of *S. fragilis* extract and incubated 30 min. at 37°. Proteins were then precipitated by adding 3 ml. of alcohol and a drop of 5 % acetic acid. After centrifugation the alcoholic solution was concentrated and deposited as a band on a 15-cm. wide Whatman No. 1 paper. Spots with glucose and glucose 1-phosphate were run at the same time. The solvent used was made by mixing 75 ml. of ethanol with 30 ml. of 1 *M* sodium acetate buffer of pH 3.8. After chromatography a portion of the paper was sprayed with aniline-phthalate reagent (6) and heated: the R_f values were: UDPG = 0.18, glucose 1-phosphate = 0.33, glucose = 0.55. From other experiments it was known that all the hexose monophosphates give about the same R_F with this solvent. Bands corresponding to the position of UDPG and of glucose phosphate were cut and extracted with water. The solutions were acidified to pH 2 with sulfuric acid and heated 30 min. at 100°. Barium carbonate was then added to neutrality and the liquids were chromatographed with the pyridine-ethyl acetate solvent. The extract from the UDPG zone was found to contain both glucose and galactose. That of the hexose phosphate zone gave traces of glucose only.

(c) *Separation of the Cyclic Phosphates*

Experiment I. The experimental technique used was the same as in the previous experiment except that the solvent for the chromatography was made by mixing 75 ml. of 95 % ethanol with 30 ml. of concentrated ammonia. The R_F values were: glucose 1-phosphate, 0.27; cyclic phosphate, 0.56, and glucose, 0.61. The glucose 1-phosphate, and the cyclic phosphate band were extracted hydrolyzed 20 min. at 100° in 1 *N* sulfuric acid, the acid neutralized with barium hydroxide, and the supernatant was chromatographed with pyridine-ethyl acetate. The extract from the diester zone was found to contain glucose and galactose, and that of the glucose 1-phosphate zone showed faint traces of glucose only.

Experiment II. In order to obtain a better separation of hexoses from the diesters a similar experiment was carried out in which the ethanol-ammonia solvent was allowed to run only 16 cm., and after drying was replaced by pyridine-ethyl acetate. On development with aniline-phosphate, three bands were visible at 7.1, 17.7, and 30.2 cm. A control with the enzymatic reaction stopped at 0-time showed the same bands. Known samples of glucose appeared at 31.7 cm.; galactose, 29,5 cm.; glucose 1-phosphate and galactose 1-phosphate, 5-10 cm. The bands (4 cm.) were extracted and hydrolyzed in order to identify the sugars as in the previous experiments.

All the samples showed the presence of glucose while galactose was found only in the hexose phosphate (7.1 cm.) and in the cyclic phosphate (17.7 cm.) zones of the incubated sample.

Summary

Treatment of uridine diphosphate glucose (UDPG) with an enzyme of S. *fragilis* was found to produce about 25 % of a galactose-containing compound. This compound is precipitated with mercuric ions like UDPG, and its migration in chromatography in acid-ethanol is similar. By alkaline treatment it gives, like UDPG, a doubly esterified hexose monophosphate. It is concluded that the compound is uridine diphosphate galactose, and the bearing of this finding on the mechanism of action of UDPG is discussed.

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CIENCIA E INVESTIGACION
Tomo 8, p. 469, Oct. 1952

URIDINA-DIFOSFATO-ACETILGLUCOSAMINA

Aislamiento e identificación

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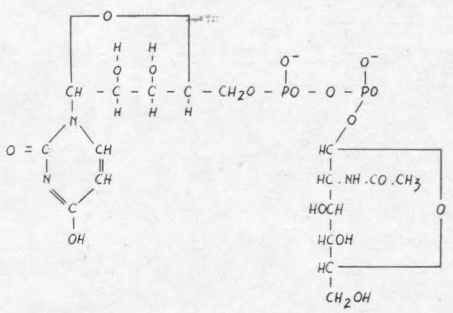
Se demostró en trabajos anteriores que la transformación del galactosa-1-fosfato en glucosa-1-fosfato requiere una coenzima: el uridina-difosfato-glucosa (UDPG) [1, 2]. Estudiando por la cromatografía sobre papel preparaciones de esta substancia obtenidas de levadura y parcialmente purificadas, se descubrió junto al UDPG la presencia de un compuesto que al parecer difería de éste por contener una substancia. (X) diferente de la glucosa [3]. Este nuevo compuesto fue designado provisoriamente UDPX, pero actualmente resulta más apropiado designarlo UDPAG.



Park y Johnson [4] observaron que substancias de estructura parecida se acumulan en el *Staphylococcus aureus* tratado con penicilina, y Park [5] ha llegado a la conclusión que uno de estos compuestos sería similar al UDPG, pero que en lugar de glucosa contendría un resto de ácido amino urónico.

Recientemente, separando los nucleótidos libres de la levadura de panadería con resinas aniónicas, se ha logrado obtener mayores cantidades de UDPAG y se han podido llevar a cabo estudios sobre su estructura.

La preparación se efectuó por extracción de levadura de panadería comercial con alcohol, seguida de precipitación con sales de mercurio y separación cromatográfica de los nucleótidos con una columna de resina Dowex-1, siguiendo en líneas generales la técnica descrita por Cohn [6]. El UDPAG contenido en las fracciones obtenidas con este tratamiento fue luego absorbido sobre carbón, eluído con alcohol acuoso amoniacal y precipitado como sal de calcio.

La substancia reductora diferente de la glucosa que se libera del UDPAG por hidrólisis ácida moderada (ácido sulfúrico 0.01 N, 10 minutos a 100ºC), fue identificada como acetilglucosamina en base a las propiedades siguientes: 1) da la reacción de Morgan y Elson [7] para acetilhexosaminas; 2) su R_f en cromatografía de papel con distintos solventes es idéntico al de la acetilglucosamina; 3) por hidrólisis prolongada con ácido sulfúrico 0.1 N se libera glucosamina, como se puede comprobar por la reacción de Elson y Morgan [8] para hexosaminas no acetiladas, y por el Rf en el papel, usando un solvente compuesto de piridina, acetato de etilo, amoníaco y agua. Como la reacción de Morgan y Elson efectuada sobre el UDPAG sin hidrolizar es negativa, la acetilglucosamina debe estar sustituida en el C 1, como la glucosa en el UDPG.

La estructura del resto de la molécula del UDPAG fue investigada con métodos similares a los utilizados para el UDPG.

La uridina se identificó y determinó cuantitativamente por su espectro de absorción en el ultravioleta. La relación uridina: fosfato: acetilglucosamina es aproximadamente 1:2:1. Las curvas de hidrólisis en ácido del azúcar y del grupo fosfato lábil del UDPAG son similares a las del UDPG. Los nucleótidos liberados por hidrólisis de 10 minutos en ácido 0.01 N y de 20 minutos en ácido 1 N a 100ºC, fueron identificados respectivamente como uridina-5'-pirofosfato y uridina-5'-fosfato por cromatografía en papel, compa-

rando con especímenes conocidos. La posición del fosfato en el uridina-monofosfato fue confirmada por tratamiento con una fosfatasa de veneno de serpiente que hidroliza específicamente los 5'nucleótidos [9].

La curva de titulación electrométrica del UDPAG muestra, como en el caso del UDPG, dos grupos fosfato primarios y ningún secundario. Un grupo fosfato secundario aparece después de hidrolizar la acetilglucosamina y otro se pone en libertad al hidrolizar el fosfato lábil.

De todas estas determinaciones se puede deducir que en el UDPAG el uridina pirofosfato está unido a la posición 1 de la acetil-glucosamina, como se ve en la fig. 1. Los datos analíticos obtenidos para una muestra de la sal de calcio fueron los siguientes (las cifras representan porcentaje en peso y aquéllas entre paréntesis son las teóricas calculadas para la fórmula de la fig. 1): nitrógeno, 6.35 (6.4); fósforo total, 8.2 (9.46); fósforo lábil (20 minutos ácido 1 N a 100ºC), 4.05 (4.73); uridina (por absorción a 260 mμ), 37.2 (36.0); acetilglucosamina, 34.4 (33.8).

Se han realizado diversos ensayos para determinar la función del UDPAG en la levadura, pero sin resultado hasta ahora. Dada la similitud de su estructura con la del UDPG se puede pensar que tenga también una función coenzimática análoga en el metabolismo de las hexosaminas.

Este trabajo será publicado próximamente "in extenso".

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BIOCHEMICAL JOURNAL
Vol. 51, p. 426, 1952

STUDIES ON URIDINE-DIPHOSPHATE-GLUCOSE

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A previous paper (Caputto, Leloir, Cardini & Paladini, 1950) reported the isolation of the coenzyme of the galactose-1-phosphate glucose-1-phosphate transformation, and presented a tentative structure for the substance. This paper deals with: (*a*) studies by paper chromatography of purified preparations of uridine-diphosphate-glucose (UDPG); (*b*) the identification of uridine-5'-phosphate as a product of hydrolysis; (*c*) studies on the alkaline degradation of UDPG, and (*d*) a substance similar to UDPG which will be referred to as UDPX.

UDPG preparations studied by chromatography. Paper chromatography with appropriate solvents has shown that some of the purest preparations of UDPG which had been obtained previously contain two other compounds, uridinemonophosphate (UMP) and a substance which appears to have the same constitution as UDPG except that it contains an unidentified component instead of glucose. This substance will be provisionally referred to as UDPX (Fig. 1 *a*).

The three components have been tested for coenzymic activity in the galactose-1-phosphate glucose-1-phosphate tarnsformation, and it has been confirmed that UDPG is the active substance. For each mole of uridine of UDPG in a sample extracted from the paper the total phosphate was 2:04, the labile phosphate (15 min. in N-acid at 100°) 1:04, and the reducing power (calc. as glucose) after hydrolysis (10 min. in 0:01 N-acid at 100°) 1:03 moles.

When UDPG is hidrolysed at pH 2 during 10 min. at 100° glucose is liberated and, as shown in Fig. 1 *b*, the UDPG and UDPX peaks are replaced by a slow-moving component which is uridinediphosphate.

Fig. 1 *c* shows the results obtained after inactivating UDPG with alkali. Besides uridine phosphate a fast-and/or a slow-moving sugar ester are formed.

Identification of uridine 5'-phosphate .The product obtained by hydrolysing off with acid the glucose and one phosphate group from UDPG was previously (Caputo *et al.* 1950) postulated to be uridine 5'-monophosphate. However, the hydrolysis curves of this compound resembled more those given by Gulland & Smith (1947) for uridine-2'-phosphate. Since then Brown, Haynes & Todd (1950) have found that the substance supposed to be uridine-2'-phosphate was uridine-5'-phosphate. The hydrolysis product of UDPG has now been compared with a synthetic specimen of uridine-5'-phosphate. Both substances were found to be identical as judged by chromatographic behaviour (Fig. 1) and by the rate of acid hydrolysis (Table 1). The crystalline barium salts of the two substances were prepared, and after recrystallization from water is was found that the microscopic aspect of both samples was the same. The X-ray diffraction patterns obtained by Prof. Galloni were identical for both samples.

TABLE 1

Acid hydrolysis of uridine phosphates

(samples heated at 100° in 0.1 N.H_2SO_4)

Time (hr.)	P hydrolysed (%)		
	Synhetic uridine-5'-phosphate	UMP from UDPG by acid hydrolysis	UMP from UDPG by alkaline hydrolysis
8.2	12.5	13.7	13.7
20.4	26.5	28.2	29.2
36.5	44.4	46.0	43.9
59.5	57.0	59.7	58.7

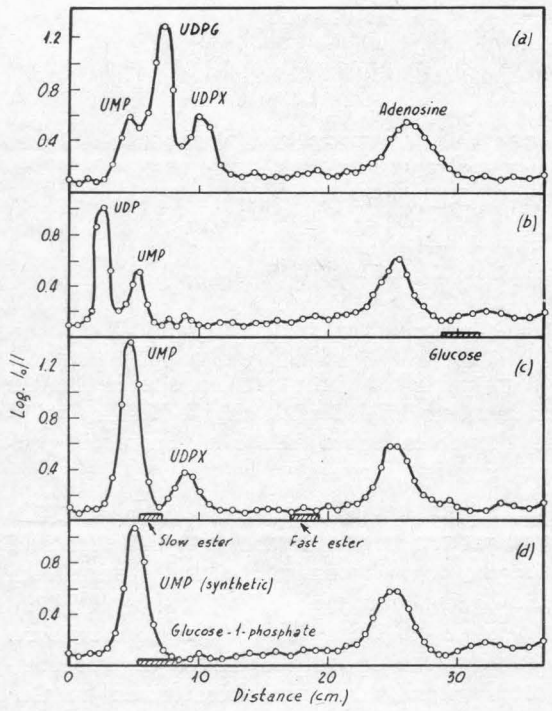

FIG. 1. — Chromatograms of UDPG preparations. Samples run simultaneously at 30°. Solvent: ethanol-M-ammonium acetate, pH 7-5. Adenosine was added as reference substance. The log I_0/I values were measured at 260 mμ *a*, partially purified UDPG; *b*, same after heating 15 min. at 100° at pH 2; *c*, heated 5 min. at 100° with excess NH_4OH; *d*, synthetic uridine-5'-phosphate plus glucose-1-phosphate. Glucose and its esters were located after removing the paraffin by ether extraction followed by spraying with aniline phtalate.

The alkaline degradation of uridine-diphosphate-glucose. It has been reported previously (Caputto *et al.* 1950) that UDPG loses its catalytic activity after mild treatment with alkali. It was found that this inactivation was accompanied by a stabilization of the glucose residue and by the liberation of a secondary acid group of phosphoric acid. Further work on this point has shown that mild alkaline treatment of UDPG leads to the formation of UMP and a glucose ester in which the phosphate is doubly esterified. This substance (Fast Ester') moves faster than any of the known glucose esters during paper chromatography. With a more drastic alkaline treatment or with acid the "Fast Ester" is transformed into another substance or substances which move more slowly. These are referred to as "Slow Ester (s)".

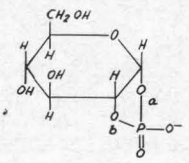

FIG. 2

The experiments which will be described can be interpreted by assigning to the "Fast Ester" the structure of a 1:2-monophosphoric ester of glucose (Fig. 2). Further treatment with alkali would yield a mixture of glucose-2-and glucose-1-phosphate by hydrolysis of the links marked *a* and *b* respectively. Treatment with acid would yield the same products, but since glucose-1-phosphate is hydrolysed immediately only glucose-2-phosphate would remain. Thus the "Slow Ester" prepared with alkali should be a mixture of glucose-1 and glugose-2-phosphates, while that prepared with acid should be glucose-2-phosphate.

The exact conditions under which UDPG is degraded with alkali have not been determined. It is decomposed rapidly during chromatography with the ethanol-ammonia solvent. Under these conditions the formation of the "Fast Ester" apparently occurs in less than 20 min., since the latter appears as a well defined spot with practically no tailing. At pH 8 at 18° UDPG remained unchanged

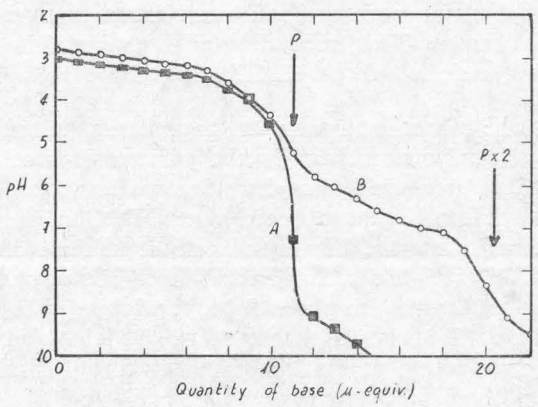

FIG. 3. — Titration curve of the 'Fast Ester'. The substance was passed through a column of cation exchange resin in the hydrogen form. One sample was titrated directly (curve *A*) and another sample (curve *B*) was heated 15 min. to 100° before titration. After this treatment 10 % of inorganic phosphate and 10 % of the glucose were liberated. The arrow marked *P* shows the μmoles of phosphate in the sample.

TABLE 2

Paper chromatography of the "fast" and "slow" esters

(Whatman no. 1 paper.)

Solvent	*Rf values*			
	'Fast Ester'	'Slow Ester'	Glucose-1-phosphate	Glucose
Ethanol (77 % v/v)	0.29	—	0.10	0.42
Ethanol ammonia	0.53	0.17	0.14	0.58
Ethanol ammonium acetate, pH 7-5	0.55	0.22	0.20	0.71

during 18 hr. At pH 8-5 in 2 min, at 100° a mixture of UMP, and "Fast" and "Slow" ester formed. In concentrated ammonia at 0° during 30 min. UMP and "Fast Ester" were formed.

Properties of the "Fast Ester". Table 2 shows the Rf values of the "Fast Ester" compared with glucose and glucose-1-phosphate. With the solvents which were used the Rf values are grossly inversely proportional to the number of acid groups in the molecule: thus hexosediphosphates move slower than the monophosphates. For the "Fast Ester" the values are nearly as high as those of free glucose. This fact was the first indication that the substance contains fewer acid groups than any of the known hexosemonophosphates. This was confirmed by electrometric titration (Fig. 3) which shows the presence of a primary but no secondary acid group. Acid hydrolysis of the "Fast Ester" yielded a sugar which was identified as glucose by paper chromatography in several solvents. The same result was obtained after hydrolysis with alkaline phosphatase.

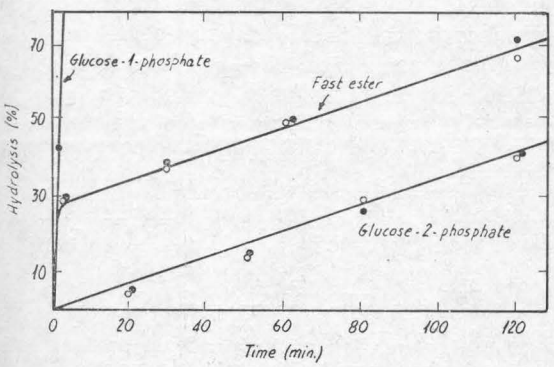

Fig. 4 — Acid hydrolysis of the 'Fast Ester'. Samples heated at 100° in 0.1 N-H_2SO_4. Reducing power measured with Somogyi's (Somogyi, 1945) copper reagent followed by arsenomolybdic acid (Nelson, 1944)
• phosphate; o reducing power as glucose.

The curve of hydrolysis of the "Fast Ester" in 0.1 N-acid is shown in Fig. 4. The curve shows a break at about 26 % hydrolysis as if it were the curve of a mixture of 26 % glucose-1-phosphate and 74 % glucose-2-phosphate. The curve of liberation of reducing power is parallel to that of phosphate liberation. With the reagent used (Somogyi, 1945) glucose-2-phosphate does not give a detectable reduction. On heating the "Fast Ester" in 0.1 N-alkali (Fig. 5) 26 % glucose-1-phosphate is formed in less than 5 min. and may be detected with the specific phosphoglucomutase test. The hydrolysis curve of the remaining 74 % of organic phosphate is similar to that of glucose-2-phosphate.

Heating the "Fast Ester" in dilute acid for a few minutes leads to the liberation of a secondary acid group of phosphoric acid (Figure 3). The change can also be detected by paper chromatography, since the "Fast Ester" is transformed into esters having, about the same *Rf* as the normal hexosephosphates (Table 2).

Osazone formation from "Slow Ester". The phosphate liberated during osazone formation was estimated on a sample of "Slow Ester" obtained from the "Fast Ester" by heating at 100° for 5 min. in 0.1 N-acid. The inorganic phosphate formed by the acid treatment was measured and subtracted from the value obtained after phenylhydrazine treatment. For comparison glucose-1-phosphate and glucose-2-phosphate were also tested. The results as follows (% liberation of P): glucose-1-phosphate, 0; glucose-2-phosphate, 94 %; "Slow Ester", 100 %.

Liberation of phosphate during osazone formation would appear in theory to be specipic for sugars with a free carbonyl conta-

ining phosphate in the 1 or 2 positions. However ,it has been observed that glucose-3-phosphate (Raymond & Levene, 1929) and fructose-3-phosphate (Levene, Raymond & Walti, 1929) also lose phosphate during osazone formation.

The structure proposed in Fig. 2 for the "Fast Ester" is consistent with experiments described in a previous paper (Leloir, 1951),

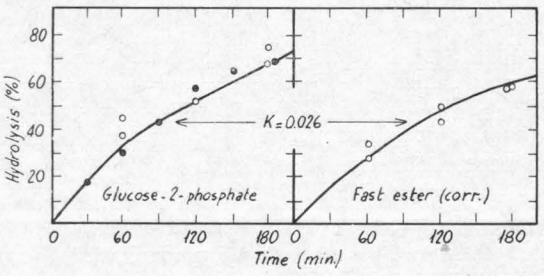

FIG. 5. — Alkaline hydrolysis of the Fast Ester'. Inorganic phosphate was estimated after samples (1.1 μM) were heated in 2 ml. of 0-1 N-NaOH to 100° in stoppered bronze tubes. In a parallel experiment glucose-1-phosphate was estimated with yeast phosphoglumutase (Cardini, Paladini, Caputto, Leloir & Trucco, 1949) activated with glucose diphosphate. Glucose-1-phosphate standards were run at the same time. The samples of the 'Fast Ester' gave 26 % of glucose-1-phosphate after heating 5 min. in 0.1 N-Na OH, and the values remained constant after heating 10 or 20 min. Glucose-2-phosphate was not affected by phosphoglucomutase. The corrected values for the percentage hydrolysis of the Fast Ester' were calculated by considering the total phosphate minus the glucose-1-phosphate as equal to 100. • Farrar's data; o this paper.

in which a "Fast Ester" containing galactose was detected besides that containing glucose. Evidence for the α structure of glucose in UDPG has been obtained from preliminary polarimetric observations. It was found that an acid treatment which hydrolysed off the glucose produced a decrease in dextrorotation: $\Delta [M] = 183°$. This value is similar to that for the conversion of α-glucose-1-phosphate to *αβ glucose* ($\Delta[M] = 218°$). For an α-glucose ester the likely positions for the formation of a cyclic phosphate would be 1:2 or 1:4. But since 1:4 would be very unlikely for an α-galactose ester it was concluded that both the glucose and the galactose esters were probably esterified at the 1 and 2 positions.

It may be mentioned that Forrest & Todd (1950) have described the formation of a cyclic phosphate of riboflavin by alkaline treatment of flavin-adenine-dinucleotide. Periodate oxidation used 1 mole of oxidant and gave no formic acid so that it was concluded that the phosphate was esterified at positions 4 and 5 of the ribityl residue.

UDPX. The 'substance giving the small peak which runs faster than UDPG (Fig. 1a) has been isolated in small amounts by paper chromatography. Analysis showed that the ultraviolet spectrum at different pH values and after bromine treatment was that of uridine, and that for each mole of uridine in UDPX the total phosphate was 2.04, labile phosphate (15 min. in N-acid at 100°) 1.0, and reducing power after hydrolysis (10 min. in 0.01 N-acid at 100°, calc. as glucose) 0.5 mole.

UDPX was found to remain unaffected by a treatment with alkali sufficient to decompose UDPG (Fig. 1 *c*).

The unknown component of UDPX has been studied by chromatography in various solvents, and it has been found to be different from the following substances: aldohexoses, pentoses, fructose, tagatose, sorbose, glucosamine, uronic acids, fucose, rhamnose, xylulose, ribulose, deoxyribose, adonese, erythrulose, 1- and 3-metryl fructose, 2- and 3-methyl glucose, glyceraldehyde and dihydroxy-acetone.

URPX was found to be clearly different from UDP galactose (Leloir, 1951) and from the compound found by Park & Johnson (1949) and Park (1950) in *Staphylococcus aureus.* The *Rf* values, both of the intact substances and of the sugars obtained by hydrolysis, were different.

The substance X was found to be unfermentable by baker's yeast and to give negative results in the following tests: resorcinol for ketoses (Roe, 1934); Elson & Morgan (1933) for amino sugars, orcinol for pentoses Mejbaum, 1939), and the test for methyl pentoses (Nicolet & Shinn, 1941). With the anilinephthalate reagent it gave a brownish-yellow colour which only appeared after prolonged heating.

EXPERIMENTAL

Methods. Analytical methods and preparations were as described in previous papers (Caputto *et al.* 1950) Glucose-2-phosphate was prepared from diphenyl 1:3:4:6-tetraacetyl-β-D-glucose-2-phosphate (Farrar, 1949) kindly supplied by Mrs K. R. Farrar. A sample of synthetic uridine-5'-phosphate was obtained from Prof. A. R. Todd.

The 'Slow Ester' was usually prepared by paper chromatography of UDPG with ethanol-ammonia as solvent. The position of the substance was revealed in a small part of the paper with aniline phthalate and the ester was subsequently extracted with water.

Paper chromatography. Descending chromatography was usually employed, except when the experiments were carried out in a thermostat. In these cases a more compact chamber similar to that described by Block (1950) was used in which the solvent travels first upwards and then downwards.

Whatman paper no. 1 was employed throughout. For nucleotides and phosphoric esters it was found convenient to wash the paper with 2N-acetic acid (Hanes & Isherwood. 1949). Usualy a pad of blotting paper was stapled at the end of the strips, and the position of the substances was referred to appropriate substances such as glucose for sugars and adenosine for nucleotides. The solvents used were: (*a*) 7-5 vol. of 95 % ethanol plus 3 vol. of M-ammonium acetate (pH-7:5; (*b*) same as (*a*) but with M-ammonium acetate buffer of pH 3:8 and (*c*) 7:5 vol. of 95 % ethanol plus 3 vol. of concentrated ammonia. With solvent (*a*) the nucleotides give values of $R_{adenosine}$ below 0:7, while the nucleotides give higher values (Table 3). The R_f values of nucleotides vary with the pH of the solvent (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950) and with temperature. As shown in Fig. 6, the changes with temperature are not parallel for all the substances.

Substance	Solvent: Ethanol-ammonium acetate, pH 7.5	Solvent: Ethanol-ammonium acetate, pH 3.8
Thymine	—	1.25
Uracil	1.13	1.16
Uridine	1.13	1.16
Cytidine	—	1.10
Adenine	1.06	1.00
Hypoxanthine	—	1.00
Adenosine	1.00	1.00
Uridine-3'-phosphate	0.46	0.94
Guanosine	—	0.88
Cytidylic acid	0.84	0.85
Uridine-5'-phosphate	0.35	0.84
Adenosine-3'-phosphate	0.29	0.76
Adenosine5'-phosphate	—	0.73
UDPX	0.55	0.73
UDPG	0.43	0.65
Guanylic acid	0.22	0.63
Uridine diphosphate	0.14	0.59
Diphosphopyridinenucleotide	0.21	0.32
Adenosinetriphosphote	0.07	—
Xanthine	0.0	0.0
Guanine	0.0	0.0

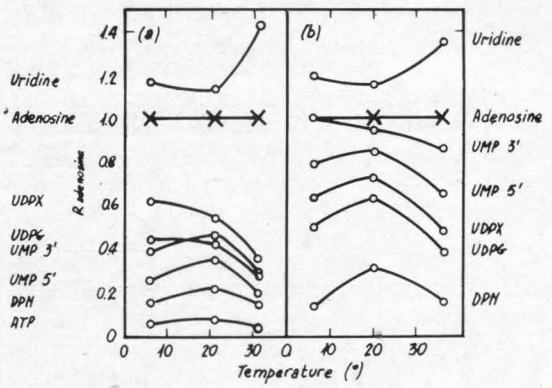

FIG. 6. — Paper chromatography of some purine and pyrimidine derivatives. Ethanol-ammonium acetate solvents. *a*, of pH 7:5, and *b*, of pH 3:8 as described in text.

The position of ultraviolet-absorbing substances was ascertained by measuring the extinction at 260 mμ after impregnation of the paper with liquid paraffin. A standard Beckmann spectrophotometer was used with an accessory which allowed strips of paper to be run along the photocell entrance (Leloir & Paladini, 1951).

Sugars and their esters were revealed with aniline phthalate reagent (Partridge, 1949).

Liberation of phosphate with phenylhydrazine. This procedure was described by Deuticke & Hollmann (1939) for the estimation of fructosediphosphate. The analytical procedure has been modified by Dr. Cardini as follows:

Reagents: (*a*) 6 % (w/v) phenylhydrazine hydrochloride in water (decolorized with charcoal if necessary); (*b*) saturated solution of sodium acetate; (*c*) saturated Na_2SO_3.

The samples and phosphate standards in 0.5 ml. of water plus 0.1 ml. of (*a*), 0.05 ml. of (*b*) and 0.1 ml. of (*c*) were heated 30 min. in a boiling-water bath. After cooling 0.75 ml. of 5 N-H_2SO_4, 0.75 ml. of 2.5 % ammonium molybdate and water to a total vol. 7.5 ml. were added. After 10 min. the optical density was measured at 660 mμ. Controls heated without phenylhydrazine were run at the same time.

SUMMARY

1. Purified preparations of uridine-diphosphate-glucose (UDPG) were studied by paper chromatography and found to be contaminated with uridylic acid and a substance UDPX.

2. The uridylic acid obtained by degradation of UDPG has been identified as uridine-5'-phosphate.

3. The alkaline degradation products of UDPG are uridine-5'-phosphate and a cyclic phosphate ester of glucose, probably esterified at positions 1 and 2 of the glucose. This ester decomposes with acid or alkali giving glucose-1-phosphate (25 %) and glucose-2-phosphate (75 %).

4. The contaminating substance UDPX appears to have the same structure as UDPG except that it contains an unidentified component instead of glucose.

The studies with synthetic uridine-5'-phosphate and with many samples of rare sugars were possible owing to the kindness of Prof. A. R. Todd, F.R.S., and the identification of glucose-2-phosphate by the generosity of Mrs K. R. Farrar. We wish to express our thanks to them as well as to Prof. E. E. Galloni for the X-ray diffraction studies, to Dr C. E. Cardini for his co-operation with the phenyl-hydrazine method and to Dr. J. T. Park for a sample of the *Staphylococcus aureus* compound.

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BIOCHIMICA ET BIOPHYSICA ACTA
Vol. 12, p. 15, 1953

THE BIOSYNTHESIS OF GLUCOSAMINE *

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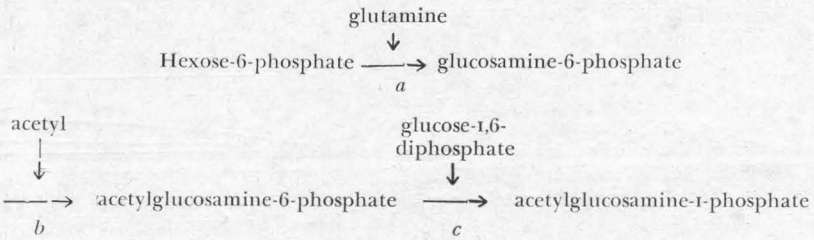
After the isolation of uridine-diphosphate-glucose (UDPG) [1], a very similar compound (UDPG) containing acetylglucosamine instead of glucose was found in yeast [2]. Considering the structural similarity of the two compounds and the coenzymic function of UDPG in the transformation of galactose-1-phosphate into glucose-1-phosphate, it has been considered that UDPG might be involved in the metabolism of hexosamine phosphates. Part of the plan of investigation consisted in a search for enzymes in some organism with a high hexosamine metabolism. Since molds should synthesize large amounts of glucosamine in order to build their cell walls, which contain chitin, experiments have been carried out with *Neurospora crassa*. While no information on a coenzymic function of UDPG has been obtained several enzymes have been found. Besides a chitinase, the *Neurospora* extracts were found to contain the enzymes required for the following sequence of reactions:



Reaction *a* and some preliminary studies on reaction *b* will be dealt with in this paper. As to reaction *c*, it has been detected by using synthetic acetylglucosamine-1-phosphate. *Neurospora* extracts were found to catalyze the transformation of this substance into acetylglucosamine-6-phosphate, but no such activity could be detected in rabbit muscle extracts. The interconversion of the acetylglucosamine phosphates is acelerated by glucose-1,6-diphosphate, a fact which bears a resemblance to its action on the mannose [3] and on the ribose-phosphates [4]. Further studies designed to decide whether the phosphoglucomutase and phosphoacetylglucosaminemutase actions are due to one or two enzymes, and to clarify the mechanism of the stimulation by glucosediphosphate are being carried out.

The synthesis of glucosamine-6-phosphate can also be brought about by a mechanism different from reaction *a*. Thus Harpur and Quastel [5] discovered that glucosamine is phosphorylated by ATP in the presence of brain extracts, and from further studies by Brown [6] and Grant and Long [7], it has been concluded that the phosphorylation is catalysed by hexokinase and that the reaction product is glucosamine-6-phosphate. It is difficult to decide whether this synthesis is a physiological process or simply an unspecific effect.

Similar events have been found to occur with galactosamine. Liver and yeast extracts containing galactokinase were found to phosphorylate galactosamine to a product which appears to be galactosamine-1-phospate [8]. In

* This investigation was supported in part by a research grant (G-3442) from the National Institutes of Health, Public Health Service and by the Rockefeller Foundation.

this case, as with glucosamine, the corresponding hexose inhibits the phosphorylation of the hexosamine.

The formation of glucosamine by a process such as reaction *a* would explain the results of TOPPER AND LIPTON [9], who found that in *Streptococcus* the glucosamine formed from glucose-1-^{14}C contained nearly all the label in the 1-position.

METHODS

Analytical. The following methods were used: BLIX[10] for glucosamine. KUNITZ AND MCDONALD [11] for protein. Glutamate was estimated with ninhydrin after paper chromatography with phenol [12]. Amide nitrogen by estimation of the ammonia liberated after heating eleven minutes at 100° in 1 *N* acid [13]. Ammonia by distillation in Conway units [14] and nesslerization.

For the estimation of acetylglucosamine the method of MORGAN AND ELSON [15] was slightly modified in order to make it less sensitive to buffers and to reduce the time needed for colour development. The *p*-dimethylamino benzaldehyde (DAB) reagent was prepared by adding 0.5 g of DAB to 10 ml of concentrated HCl and completing to 100 ml with glacial acetic acid. The analytical procedure was as follows: the neutralyzed unknowns and standards containing 0.1-0.5 μmoles of acetyl glucosamine were taken to 0.5 ml with water. After adding 0.1 ml of 1 *M* sodium carbonate the tubes were heated 5 minutes in a boiling water bath. After cooling 2.5 ml of the DAB reagent were added and mixed immediately with a suitably glass rod. The optical density at 544 mμ was measured after 3 to 5 minutes with a Beckman spectrophotometer. The colour increases during 2 minutes and begins to decrease slowly after 3 minutes. If the time elapsing between the addition of the DAB reagent and the colorimetric reading is equal in all the samples a good proportionality between concentration of acetylglucosamine and optical density is obtained.

Preparation of the enzyme. A wild type *Neurospora crassa* E-5297a was grown for three days on "minimal medium" [16] at 30° under forced aeration. The mycelium was separated by filtration, washed with water, lyophylized and stored over calcium chloride in an evacuated dessiccator at 5°. Extraction of the dried mycelium was effected by homogenizing 0.8 g in 16 ml of water at 0°, followed by centrifugation. The supernatant containing about 40 mg of protein per ml is referred to as crude extract.

Partial purification was carried out as follows: 6.5 ml of acetone were added to 13 ml of the crude extract at 0°. The inactive precipitate was centrifuged off at 0°. To the supernatant 3.9 ml of acetone were added. The precipitate was separated by centrifugation, washed three times with acetone and dried in an evacuated desiccator. The yield was about 60 mg of a white powder.

The formation of "glucosamine" was found to be greater in the presence of 8-hydroxyquinoline, and this fact was attributed to protection of the enzyme from metal inactivation. Therefore, 8-hydroxyquinoline was added to the acetone used in the purification (about 10 mg %) and the buffer (pH 6.5) used for dissolving the enzyme was saturated with 8-hydroxyquinoline.

The enzyme in solution was found to lose activity in a few hours at 5° and in a few days at −10°.

The ratio: μmoles of glucosamine formed/mg protein per hour, was about 0.04 for the crude enzyme and usually about 0.3 for the acetone fractionated enzyme.

Acetylation experiments. The enzyme preparation used was a crude extract which had been dialyzed about two hours against running water. The enzyme system was similar to that used by KAPLAN AND LIPMANN [17]. The CoA solution was an aqueous extract of rat liver. In every case controls in which the reaction was stopped at time = 0 were run simultaneously. The reaction was stopped by immersing the tubes in boiling water followed by centrifugation.

Acetylglucosamine was estimated in the supernatant as described above. In some cases the phosphoric esters were precipitated by adding 0.3 ml of 5 % zinc sulphate and 0.3 *N* barium hydroxide until the suspension gave a rose colour with phenolphthalein.

The formation of "glucosamine". Incubation of hexose-6-phosphate with glutamine and the enzyme gave rise to an increase in the glutamine content. As shown in Fig. 1, hardly any increase took place when glutamine or hexose-6-phosphate were omitted.

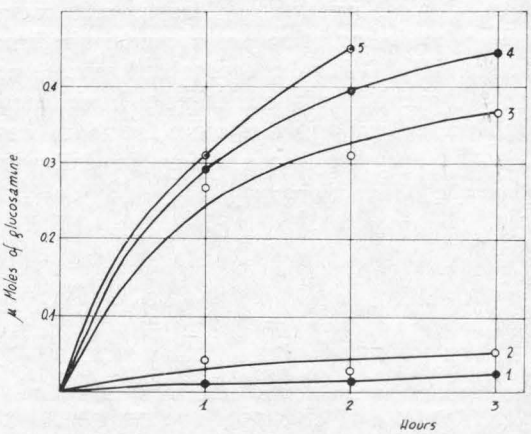


FIG. 1. — The formation of glucosamine. Incubation at 30° of 1 mg of purified enzyme and 0.05 ml of trishydroxymethylaminoethane acetate buffer of pH 6.4 with substrates. Total volume, 0.2 ml.

Curve 1, 0 μmoles of hexose-6-phosphate + 2 μmoles of glutamine
Curve 2, 2 μmoles of hexose-6-phosphate + 0 μmoles of glutamine
Curve 3, 1 μmoles of hexose-6-phosphate + 5 μmoles of glutamine
Curve 4, 4 μmoles of hexose-6-phosphate + 2 μmoles of glutamine
Curve 5, 4 μmoles of hexose-6-phosphate + 5 μmoles of glutamine

The results of an analysis of the chemical changes occurring during the reaction appear in Table 1. The increase in "glucosamine" was approximately equal to the decrease in amide nitrogen of glutamine and to the increase in glutamate. There occurred also a small increase in ammonia in the complete system as well as in the controls without glutamine.

An experiment carried out at different temperatures appears in Table II. At 37° glucosamine formation was faster than at 30° in the beginning, but slower afterwards.

In many experiments it was observed that the enzyme solutions became rapidly inactivated at room temperature. On the other hand, in the experiments of Fig. I the enzyme in the presence of the substrates was still active after 3 hours at 30°. An experiment was therefore carried out in order to ascertain which of the substrates exerted a stabilizing action. Samples of the enzyme were preincubated 30 minutes at 30° with or without substrate, and then the enzyme system was completed.

The glucosamine formed in one hour was as follows (the amount formed during preincubation was subtracted):

Preincubated without substrate	0.50
Preincubated with glutamine	0.76
Preincubated with hexose-6-phosphate	0.70
No preincubation	0.80

Thus both substrates, and specially glutamine, exerted a considerable stabilizing action.

Specificity. Glucose-6-phosphate could be replaced by fructose-6-phosphate, but not by any of the following substances: maltose, glucose, mannose, fructose, fructose-1,6-diphosphate, glucose-1,6-diphosphate, α-galactose-1-phosphate, fructose-1-phosphate, glucose-2-phosphate, xylose-5-phosphate, dihydroxyacetone or glyceraldehyde.

The enzyme preparation was found to contain considerable amounts of the enzyme which catalyzes the interconversion of fructose-6-phosphate into glucose-6-phosphate. The activity of this isomerase was estimated by measuring the disappearance of fructose phosphate with ROE's [18] method. It was found that under the conditions used for measuring glucosamine formation the equilibrium values for the glucose-fructose esters was attained in about 5 minutes. Therefore, it has not been possible to decide whether the reactant

TABLE I

Balance experiment

The aliquots of the complete system taken for analysis contained: 1,3 μmoles of glutamine, 1.3 μmoles of glucose-6-phosphate and 0.6 mg of purified enzyme and citrate buffer. pH 6.4. Total volume, 0.1 ml. Incubated 3 hours at 30°.

	Δ *"glucosamine"*	Δ *amide*	Δ NH_3	Δ *glutamate*
Complete system	0.42	−0.39	0.17	0.36
No glutamine	0.03	−0.07	0.14	0.08
No hexose-6-phosphate	0.03	−0.04	0 22	0.10

TABLE II

Formation of "glucosamine" at different temperatures
Complete system as in Table I. Results in μmoles.

	Time of incubation (minutes)			
Temperature	30	60	120	180
24°	0.08	0.20	—	0.46
30°	0.13	0.24	0.40	0.52
37°	0.16	0.20	0.34	0.40

is glucose-6-phosphate or fructose-6-phosphate.

"Glucosamine" was formed when glucose-1-phosphate was used instead of hexose-6-phosphate with the crude enzyme, but not with the purified preparations. Under the conditions of the test and with the purified enzyme the phosphoglucomutase activity was very weak.

The substances which were tested with negative results as possible substitutes for glutamine were the following: asparagine, glutamic and aspartic acids, arginine, putrescine, urea, ammonium acetate, alanine, glycine, butyramide, serine, cysteine, lysine, ornithine, valine, leucine and citrulline. Pairs such as ammonium salts with ATP, asparagine and glutamate, etc., also gave negative results.

pH optimum. As shown in Fig. 2, the reaction has a sharp pH optimum at pH 6.4-6.8.

Study of the "glucosamine" ester. 100 μmoles each of glutamine and hexosemonophosphate plus 50 mg of enzyme in 10 ml of 0.025 *M* tris-acetate buffler (pH 6.4) were incubated 3 hours at 30°. The proteins were removed by heat coagulation. Barium acetate was added to the clear liquid and the pH was adjusted to 8. The mixture was centrifuged and the precipitate was washed twice with 1 ml of water. Three volumes of ethanol were added to the pooled supernatants. The precipitate was redissolved in 10 ml of water, a small precipitate centrifuged off and three volumes of ethanol were again added. The precipitate was then dried with ethanol and ether. Yield, 20 mg. These were dissolved in 2 ml of water. The solution contained 41 μmoles of total phosphate, 36 μmoles of reducing substance calculated as glucose, and 5.5 μmoles of "glucosamine". Direct paper chromatography of this ester mixture in different solvents gave irregular results, so that it was decided to remove the phosphate group.

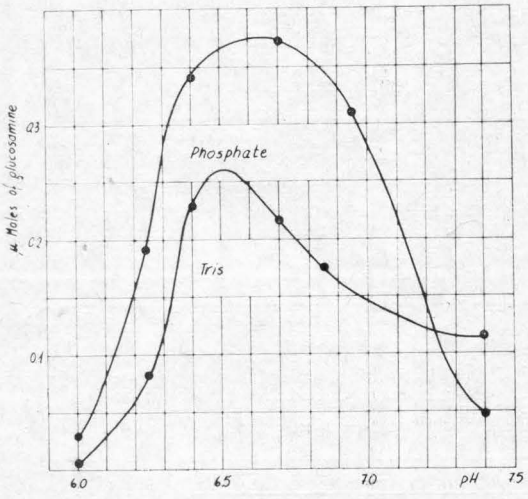


FIG. 2. — pH optimum curve. System composed of 2 μmoles each of hexose-6-phosphate plus 1 mg of enzyme and 0.1 ml of 0.1 *M* phosphate or trishydroxymethylaminoethane acetate buffers. Incubated 2 hours at 30°. The pH was determined on aliquots with a glass electrode.

0.5 ml of the above solution was made 0.01 *M* in respect to Mg^{+2}, and 10 mg of a kidney phosphatase preparation and a drop of toluene were added. After 16 hours at 37°, about 70 % of the phosphate was hydrolysed. The mixture was then deproteinized with trichloroacetic acid, washed with ether and used for paper chromatography. A sample of glucosamine-6-phosphate was run simultaneously. One of the solvents used was a mixture of ethyl acetate-pyridine-ammonia [2] with which it is possible to separate glucosamine from galactosamine. The other solvent was phenol-water [19] with ammonia. Phenol without ammonia was used with paper which had been immersed in 0.1 *M* zinc sulphate and dried in air. This procedure was based on a previous observation which disclosed that zinc ions greatly retard the migration of hexosamines but only have a small influence on others sugars. It was also observed that with an alkaline solvent there was no retardation by zinc ions.

The results of the chromatography are shown in Table III. The ex-ester sugar mixture gave spots which migrated like glucose and glucosamine. The position of the substances was revealed with the aniline phthalate reagent [20], and that of hexosamines was checked with the modified ELSON AND MORGAN reagent [19]. Besides glucose and glucosamine, the ex-ester mixture contained small amounts of another hexose which migrated like mannose. In some cases a very faint spot with the *R-glucose* value of fructose was observed. The presence of these sugars is not surprising since the sample of hexose-6-phosphate used was obtained by the action of yeast enzymes.

TABLE III

Paper chromatography of the "glucosamine" ester after treatment with phosphatase

	Ethyl acetate-pyridine-NH_3	*Rglucose Phenol-NH_3*	*Phenol-SO_4Z treated paper*
Ex-"glucosamine" ester	0.99, 0.62, 1.39	1.0, 1.73, 1.54	1.01, 0.13, 1.19
Ex-glucosamine-6-phosphate *	0.61	1.69	0.14
Glucosamine	0.61	1.69	0.14
Galactosamine	0.36	1.87	0.15
Fructose	1.42	1.47	1.45
Mannose	1.35	1.47	1.15

* Prepared from glucosamine with ATP and hexokinase 6.

Acetylation. As shown in Table IV, *Neurospora* extracts, when suitably supplemented are able to bring about the acetylation of glucosamine. These extracts are also able to catalyze the phosphorylation of glucosamine (Table V) and contain phosphatase. The result of the action of this set of enzymes is that starting with free glucosamine or with glucosamine-6-phosphate, the reaction products are similar. Most of the acetylglucosamine appears free and a part precipitates with zinc suplphate-barium hydroxide as would acetylglucosamine phosphate (Table VI). If acetylglucosamine phosphate was formed, it could not have arisen by phosphorylation of acetylglucosamine, since this process is not catalyzed by the extracts (Table V). Therefore, it seems logical to conclude that gluco-

TABLE IV

The acetylation of glucosamine

Complete system: 2 μmoles of glucosamine, 4 μmoles of ATP, 0.065 ml of 1 *M* sodium acetate, 0.05 ml of 0.1 *M* cysteine, 0.05 ml of 0.2 *M* sodium citrate of pH 7, 0.1 ml of CoA solution, 0.05 ml of 0.1 *M* magnesium chloride and 0.2 ml of crude dialyzed enzyme. Final volume, 0.7 ml. Incubation 2 hours at 37°.

	μ moles of acetylglucosamine formed
Complete system	0.38
No glucosamine	0.025
No CoA	0.075
No ATP	0.025
No Mg++	0.070

TABLE V

The phosphorylation of glucosamine

Complete system as in Table III, but without CoA. The difference in glucosamine or acetylglucosamine content between samples incubated with and without ATP was considered to be due to phosphorylation. The estimations were carried out after precipitation of proteins and phosphoric esters with zinc sulphate and barium hydroxide.

Substrate	*μ moles of substrate phosphorylated*	
	No Mg ++	*With Mg ++*
Glucosamine	0.8	1.80
Acetylglucosamine	0	0

TABLE VI

The acetylation of glucosamine phosphate

Complete system as in Table III. The acetylglucosamine content of the supernatants after zinc sulphate-barium hydroxide precipitation was considered as free acetylglucosamine.

Substrate	*Time of incubation (minutes)*	*μmoles of acetylglucosamine formed*	
		Total	*Free*
Glusosamine	30	0.26	0.18
	60	0.40	0.28
	120	0.38	0.25
Glucosamine-6-phosphate	120	0.60	0.49

TABLE VII

The acetylation of the "glucosamine" ester

The "glucosamine" ester was obtained by incubation during 3 hours at 30° of 2 μmoles each of glutamine and hexose-6-phosphate with the purified enzyme. The controls contained the same substances plus glucosamine (0.5 μmoles) or glucosamine-6-phosphate (1 μmol), and the reaction was stopped at $t = o$. The acetylating system described in Table III was then added.

	μ moles of acetylglucosamine formed
"Glucosamine" ester	0.21
Control with glucosamine	0.20
Control with glucosamine-6-phosphate	0.31

samine-6-phosphate can be acetylated directly to acetylglucosamine phosphate.

Table VII shows the results of the action of the acetylating system on the "glucosamine" ester formed from hexose phosphate and glutamine. This substance gave rise to acetylglucosamine, as did glucosamine or glucosamine-6-phosphate.

Discussion

The substance formed from hexose-6-phosphate and glutamine gives the Elson and Morgan and Dische [21] reactions for hexosamines. It can be prepared as the barium salt admixed with hexosemonophosphates. It behaves like glucosamine-6-phosphate when incubated with the acetylating system of *Neurospora,* and after dephosphorylation with phosphatase glucosamine can be identified by paper chromatography with selected solvents.

All this is considered as evidence proving that the product is glucosamine phosphate. The ester gives positive reactions for hexosamines, so that a 1-ester can be excluded, and since a migration of the phosphate during the reaction appears unlikely, the product should be glucosamine-6-phosphate.

No evidence for the necessity of a cofactor for the formation of glucosamine phosphate was obtained. Thus, no stimulation was obtained by the addition of different ions, ATP, pyridoxal phosphate, UDPAG, etc. The mechanism of the reaction cannot be discussed until it is decided whether the reactant is fructose-6- or glucose-6-phosphate, In order to settle this point it will be necessary to obtain enzyme preparations free from isomerase.

Further investigation will also be necessary in order to decide whether the acetylation step takes place on free glucosamine, on glucosamine-6-phosphate or on both. In connection with this point, it may be mentioned that Chou and Soodak [22] extrated an enzyme from pigeon liver which catalyzed the acetylation of free glucosamine and galactosamine, but that the corresponding phosphates were not tested.

SUMMARY

A partially purified enzyme has been prepared from *Neurospora crassa* which catalyzes the formation of glucosamine phosphate from hexose-6-phosphate and glutamine. The glucosamine phosphate was identified by colour reactions, by dephosphorylation and paper chromatography and by its behaviour towards an acetylating system.

Quantitative analysis of amide nitrogen, glutamate, and hexosamine agreed with the following equation:

Hexose-6-phosphate + glutamine → glucosamine-6-phosphate + glutamate.

Crude *Neurospora* extracts were found to phosphorylate glucosamine in the presence of ATP and, when suitably supplemented, to acetylate glucosamine or glucosamine phosphate.

RÉSUMÉ

Les auteurs out préparé et partiellement purifié à partir de *Neurospora crassa*, un enzyme qui catalyse la formation de glucosamine phosphate à partir d'hexose-6-phosphate et de glutamine. Le glucosaminephosphate a été identifié par ses réaction colorées, par la déphosphorylation, par la chromatographie sur papier et par son comportement en présence d'un système acétylant.

L'analyse quantitative de l'azote amidé, du glutamate et de l'hexosamine est en accord avec l'équation suivante:

Hexose-6-phosphate + glutamate → Glucosamine-6-phosphate + glutamate.

Les extraits bruts de *Neurospora* phosphorylent la glucosamine en présence d'ATP et écatylent la glucosamine et le glucosaminephosphate, quand on les supplémente convenablement.

ZUSAMMENFASSUNG

Ein teilweise gereinigtes Enzym, das die Bildung von Glucosaminphosphat aus Hexose-6-phosphat und Glutamin katalysiert, wurde aus *Neurospora crassa* dargestellt. Das Glucosaminphosphat wurde durch Farbreaktionen, Desphosphorylierung und Papierchromatographie und durch sein Verhalten gegenüber einem acetylierten System identifiziert.

Die quantitative Analyse von Amidstickstoff, glutaminsaurem Salz und Hexosamin stimmte mit der folgenden Gleichung überein:

Hexose-6-phosphat + Glutamin → Glucosamin-6-phosphat + Glutaminsaures Salz.

Es wurde gefunden, dass rohe *Neurospora*-extrakte Glucosamin in Gegenwart von ATP phosphorylieren und, wenn sie geeignet ergänzt werden, Glucosamin oder Glucosaminphosphat acetylieren.

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 45, p. 55, 1953

ENZYMIC PHOSPHORYLATION OF GALACTOSAMINE AND GALACTOSE [1]

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Introduction

Harpur and Quastel [1] observed that glucosamine is phosphorylated on incubation with brain extracts and adenosine triphosphate (ATP), and that glucose acts as a competitive inhibitor. From further study by Brown [2] and by Grant and Long [3] of this phosphorylation, using purified yeast hexokinase, it has been concluded that glucose and glucosamine phosphorylation are catalyzed by the same enzyme.

In the course of studies on hexosamine metabolism, it was observed that crude extracts of liver, and also of brain, catalyze a transference of phosphate from ATP to galactosamine. After partial purification of the liver enzyme, the phosphorylation of galactose could be clearly detected. The latter process is difficult to study with crude extracts, but a small activity had been reported by Bacila [4].

An extract containing an enzyme catalyzing the phosphorylation of galactose (galactokinase) can be obtained from yeast [5-6] and is more active than that of animal tissues. Therefore, it was tested with galactosamine as substrate. Phosphorylation was found to take place, and the extracts of yeast adapted to galactose were found to be more active than those from nonadapted yeast.

Experimental

Liver Enzyme

An adult rat was fasted for 36-48 hr. The liver (about 6 g.) was washed with ice water and homogenized in 2 vol. of 1 % potassium chloride. The homogenate was then centrifuged in the cold for 15-20 min. at 15,000 r.p.m. The supernatant was filtered through cotton wool to remove the floating fatty material. Half a volume of saturated ammonium sulfate solution was added, and the mixture was centrifuged and filtered as in the previous step. Half a volume of saturated ammonium sulfate was again added to the supernatant, and after centrifugation the precipitate was dissolved in 1.5 ml. of distilled water and dialyzed for about 2 hr. in the cold. The protein content of these extracts was about 70-80 mg./ml.

Extracts from nonfasted rats could be used for measuring galactosamine phosphorylation, but not for that of galactose because a considerable amount of reducing substances was liberated during incubation. After fasting, this blank was greatly reduced or suppressed in some cases.

Brain Enzyme

Rat brains were homogenized in 10 vol. of cold acetone. After washing with acetone and ether, the extract was allowed to dry at room temperature. The dry powder (500 mg.) was homogenized in 3 ml. of 0.06 *M* disodium phosphate. After 30 min. in the cold, the mixture was centrifuged and the precipitate rejected.

Yeast Enzyme

Saccharomyces fragilis was grown in yeast extract-agar containing 2 % glucose or lactose. The cells were harvested after 48 hr. at 30°, washed, extended in 2 mm. layers, and allowed to dry in air. After 3-4 days, the dry material was extracted at 5° as described by Neuberg and Lusing (7). The protein content was then adjusted to about 70 mg./ml.

Substrates

Galactosamine was prepared as described by Levene (8) from hogs' nasal septa. Adenosine triphosphate was obtained according to Needham (9).

Analytical Methods

Galactosamine was estimated by the method of Elson and Morgan as described by Blix (10). Other methods were as follows; Roe (11) for fructose; Fis-

[1] This investigation was supported in part by a research grant (G-3442) from the National Institutes of Health, U. S. Public Health Service; and by the Rockefeller Foundation.

ke and SubbaRow (12) for phosphate; Kunitz and McDonald (13) for protein; Somogyi (14) and Nelson (15) for reducing sugars. The amount of sugar phosphorylated was measured by precipitating the proteins and phosphoric esters with 0.3 *N* barium hydroxide and 5 % zinc sulfate (5, 14). Either 0.2 or 0.4 ml. of each solution was used for the test system as described in Table I. The difference in free sugar between the incubated and nonincubated samples was considered to be due to phosphorylation. Blanks without added sugar or ATP were run in some cases.

An alternative method was used in the experiment shown in Fig. 3. In this case the formation of galactosamine esterified was measured instead of the disappearance of free galactosamine. The phosphoric esters were precipitated with zinc sulfate and barium hydroxide, the precipitate was washed, and then the galactosamine phosphate was hydrolyzed with acid and the galactosamine was estimated as usual. This procedure is more laborious than the other and can only be applied when the reaction product is the 1-ester, but it has the advantage that it can be used even with high sugar concentrations.

The details were as follows: The reaction mixture was as shown in Fig. 3. A blank with no substrates and another without incubations were run at the same time. The reaction was stopped by adding 0.2 ml. each of zinc sulfate and barium hydroxide. After centrifugation, the precipitate was finely suspended in 0.5 ml. of water and centrifuged. After repeating the washing three times, the precipitate was resuspended in 0.2 ml. of water plus 0.1 ml. of 5 *N* sulfuric acid. The mixture was heated for 15 min. at 100° cooled and neutralized with sodium hydroxide. Then 0.1 ml. each of barium hydroxide and zinc sulfate was added. After mixing and centrifuging, an aliquot of the supernatant was taken for the analysis of hexosamine.

Results

Phosphorylation by Liver Extracts

The phosphorylation of galactosamine could be regularly observed with crude liver homogenates prepared in water or saline solution. The activity of the supernatants obtained by high-speed centrifugation was still greater than that of the whole homogenates. After a few hours at room temperature the activity disappeared completely. At 10° the crude or purified extracts became inactive after 3 4 days. Addition of cysteine did not affect the activity.

As shown in Table I, the purified extracts catalyze the phosphorylation of galactose and galactosamine to an equal extent and also that of fructose. The action on glucose is small and decreases with longer times of incubation, probably owing to the action of phosphatase. A weak action on glucosamine was also detected. Tests for other enzymes in these extracts revealed a weak phosphoglucomutase and glucose-6- phosphatase, and no action on galactose 1-phosphate, even after the addition of uridine diphosphate glucose (UDPG)[16].

The pH optimum curves for the phosphorylation of galactose and galactosamine appear in Fig. 1. The galactose curve shows a sharp peak at pH 7.7, while the galactosamine curve shows a broad maximum at pH 7.7 while the galactosamine curve shows a broad maximum at pH 7.3. The curve for the optimum concentration of magnesium ions appears in Fig. 2. The curves obtained with or without fluoride are nearly equal. Maximal activity was obtained at about 0.01 *M* concentration.

Crossed Inhibition with the Liver Enzyme

An experiment on the phosphorylation of galactose and galactosamine mixtures is shown in Table II. For comparison, the phosphorylation of fructose, which is known to be catalyzed by a specific enzyme[17-19], was also studied. Estimations of fructose and galactosamine were carried out by the resorcinol and Elson-Morgan methods, respectively, and those of galactose by copper reduction and by difference in the mixed-sugar tests. Therefore, the results were very accurate.

TABLE I

Phosphorylation by Purified Liver Extracts

Incubation at 37° of 0.5 μmole sugar, 2 μmoles ATP, and 0.1 ml. of 0.1 *M* disodium phosphate. Magnesium and fluoride ions at 0.01 *M* and 0.05 *M* final concentration, respectively; 0.05 ml. of purified liver enzyme. Total volume, 0.3 ml, pH 7.4.

	Per cent of sugar phosphorylated Time of incubation, min.				
Substrate	10	20	40	60	120
Galactose	43	64	73	81	25[a]
Galactosamine	44	68	83	84	12[a]
Fructose	—	45	90	—	
Glucose	—	29	20	—	

a With 0.2 ml. of brain extract instead of liver enzyme.

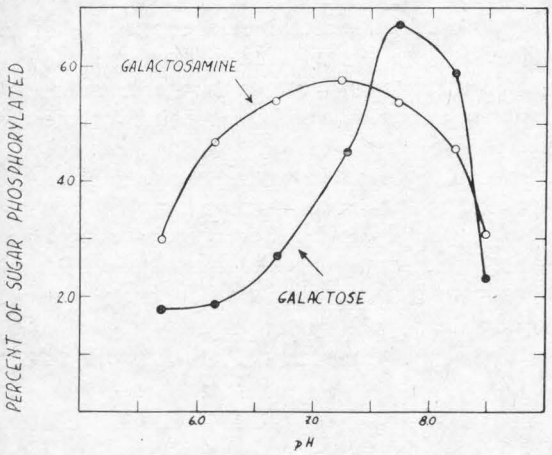

FIG. 1. — pH curve with the liver enzyme: 0.5 μmole sugar, 2 μmoles ATP, 0.05 ml. enzyme, 0.10 ml. phosphate, 0.1 *M*; $MgCl_2$ and NaF in final concentration of 0.01 and 0.05 *M*, respectively. Final volume, 0.30 ml. The pH was determined aliquots with glass electrode; 30 min. at 37°.

The results in Table II show that the rates of phosphorylation of fructose and galactosamine were approximately equal when the sugars were incubated separately or in mixtures. In the galactose-galactosamine mixtures, phosphorylation was clearly slower than in the single-sugar experiments. The inhibition of galactosamine phosphorylation by galactose,

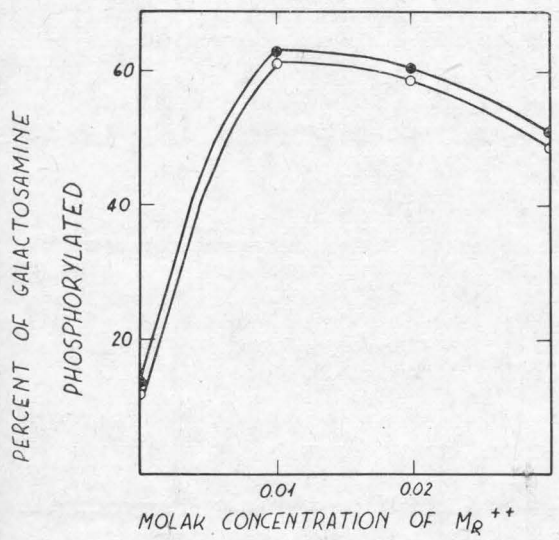

FIG. 2. — The effects of Mg++ concentration on the liver enzyme: 0.5 μmole galactosamine, 2 μmoles ATP, 0.025 ml. of liver enzyme. Final volume, 0.20 ml.; 30 min. at 37°. Lower curve with 0.05 *M* fluoride; upper curve: no fluoride.

TABLE II

Phosphoryation of Sugar Mixtures by the Liver Enzyme

Incubation at 37° of 1 μmole sugar, 4 μmoles ATP, 0.1 ml. of purified enzyme, and 0.1 ml. of 0.1 *M* phosphate buffer of pH 7.2. Magnesium and fluoride ions at 0.01 *M* and 0.05 *M* final concentration, respectively. Final volume, 0.45 ml. The test with sugar mixtures contained 0.5 μmole of each sugar.

	Per cent phosphorylation			
	Sugars by themselves		Mixed sugars	
Time of incubation, min	15	30	15	30
Galactosamine	42	64	30	62
Fructose	42	62	42	55
Galactosamine	43	72	0	0
Galactose	30	43	20	30

which is very marked, can be observed also in Fig. 3. Galactosamine phosphorylation was completely inhibited when the ratio of galactosamine to galactose was lower than about 4. On increasing the radio to about 8, some galactosamine was phosphorylated, but the inhibition was still about 80 %.

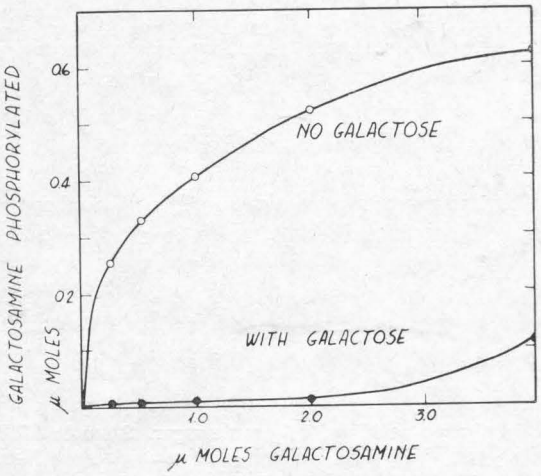

FIG. 3. — Galactosamine concentration curve and the inhibition by galactose 0.25 μmole galactose, 0.25-4.0 μmoles galactosamine, 2 μmoles ATP 0.05 ml of liver enzyme $MgCl_2$ and NaF in 0.01 M and 0.05 M respectively, final concentration; 30 min. at 37°. Total volume, 0.3 ml.

Phosphorilation by Extracts from Other Organs

The action of brain extracts on both galactosamine and galactose was found to be weak but reproducible, especially in the case of galactosamine. The effect on galactose was

only detected in tests carried out at a pH 7.7. The results shown in Table I were obtained with an extract of brain dried with acetone. With homogenates the resuls were irregular. No activity on galactosamine could be detected with extracts of kidney or intestinal mucosa.

Phosphorylation with Yeast Extracts

Extracts of brewer's yeast which do not catalyze galactose phosphorylation at a detectable rate, were found to be devoid of action on galactosamine, whereas extracts of *S. fragilis,* which contain galactokinase were found to catalyze also galactosamine phosphorylation. An experiment was carried out in which extracts prepared from. *S. fragilis* grown in glucose or in lactose were compared in respect to their kinase activity. These results are shown in Table III. Galactokinase activity was nearly 20-fold greater in the extract from the lactose-grown cells, as compared with that obtained from cells grown on glucose. The activity on galactosamine was also greater in extracts from lactose-grown cells. Accurate values for the ratio of activity on galactose and galactosamine in the two types of extracts could not be obtained owing to the low activity in the extract from glucose grown cells.

The Reaction Product

Preliminary information on the type of ester formed was obtained from an experiment in which the reaction mixture was deproteinized whit trichloroacetic acid instead of zinc sulfate-barium hydroxide. The former reagent does no precipitate phosphoric esters as does the latter. Therefore, if the reducing group of the reaction products were blocked, the same decrease in amino sugar should be obtained with both procedures. This was found to be the case. Moreover, as shown in Table IV, after acid hydrolysis of the trichloroacetic acid filtrate, most of the amino sugar was set free.

TABLE IV

Acid Hydrolysis of the Final Reaction Mixture

Incubation at 37° for 30 min. of 2 μmoles galactosamine, 10 μmoles ATP, 0.05 ml. of 0.9 *M* sodium fluoride, 0.08 ml. of 0.1 *M* magnesium chloride, and 0.2 ml. of purified liver enzyme. Final volume, 0,7 ml. Deproteinization with 20 % trichloroacetic acid followed by estimation of galactosamine before and after hydrolysis with 1 *N* hydrochloric acid at 100° for 15 min.

Time of incubation *min.*	Galactosamine No hydrolysis %	percentage Hydrolized %
0	100	95
30	28	88

Nearly pure preparations of the reaction product were obtained from larger-scale experiments in which the sugar esters were purified by precipitation of the barium salts with ethanol and by removal of adenosine phosphates with mercuric nitrate. The procedure was essentially as described by Brown [2]. The

TABLE III

Phosphorylation with Extracts of Saccharomyces fragilis

Incubation at 37° of 0.5 μmole sugar. 2 μmole ATP, Mg++ at 0.01 M final concentration, and extracted of S. *fragilis* grown on lactose or on glucose. Final volume, 0.15 ml. Both enzyme solutions contained the same concentration of protein.

Sugar in growth medium	Enzyme dilution	Amount of enzyme *ml.*	Substrate	Times of incubation, min. 10	20	30	Activity *
				Micromoles of sugar phosphorylated			
Lactose	None	0.01	Galactosamine	0.035	0.09	0.22	0.75
Lactose	1/50	0.03	Galactose	0.32	0.39	0.46	53
Lactose	1/500	0.05	Glucose	0.25	0.31	0.5	250
Glucose	None	0.01	Galactosamine	0.045	0.045	0.06	0.2
Glucose	1/50	0.03	Galactose	0.035	0.035	0.05	2.8
Glucose	1/500	0.05	Glucose	0.35	0.5	0.5	350

* Expressed in micromoles phosphorylated/min./ml. of undiluted enzyme solution.

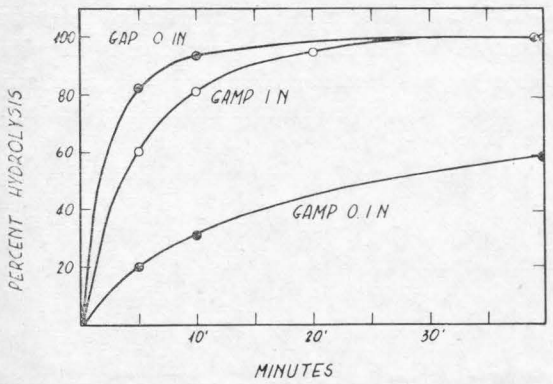

Fig. 4. — Hydrolysis curve of the galactosamine ester. Liberation of phosphate after heating at 100° in acid. GAMP: galactosamine ester. GAP: galactose 1-phosphate.

product thus obtained from galactosamine using the liver enzyme contained no inorganic phosphate and gave no Elson-Morgan reaction. After hydrolysis in 1 *N* acid at 100° for 15 min., equal amounts of phosphate and amino sugar were set free.

The product obtained from galactose using the liver enzyme was nonreducing and gave by acid hydrolysis equal amounts of phosphate and reducing sugar. Thus it appears to be galactose 1-phosphate.

Products with the same properties were obtained using extracts of *S. fragilis*. Since smaller amounts of enzyme were required, less extraneous material was introduced, and the products were purer. The acid hydrolysis curve of the galactosamine ester is shown in Fig. 4. The same results were obtained by estimating phosphate or galactosamine.

The hydrolysis of the galactosamine ester is much slower than that of the galactose ester Even in 1 *N* acid it does not reach the rate obtained with galactose phosphate in 0.1 *N* acid.

Discussion

The problem of whether galactosamine phosphorylation is brought about by galactokinase or by a specific enzyme cannot be answered with certainty, although the evidence is in favor of a single enzyme. With the liver enzyme there is crossed inhibition, the relative activities are approximately equal in different samples of liver, and both activities disappear on storage at about the same rate. Moreover, the phosphate group appears to be introduced in the 1-position in both cases. The pH-optimum curves are different, but this does not prove that two enzymes are involved. With the liver enzyme both activities are approximately equal at pH 7.5, whereas with the *S. fragilis* enzyme, the activity on galactose is higher than on galactosamine.

In *S. fragilis* the galactosamine activity increases during growth on a galactose-containing medium (lactose), but even this is no proof for a single enzyme, since in other organisms it has been found that substances different from the substrate may induce the formation of the enzyme [20].

If it is accepted that galactosamine phosphorylation is catalyzed by galactokinase, the results would be of interest because the measurement of galactosamine phosphorylation would give an estimate of galactokinase activity. This estimate cannot be obtained with galactose in crude liver extracts owing to the interference by other reducing substances. Since there is no such interference when using galactosamine, it would be possible to compare the galactokinase activity of liver samples in cases where galactose metabolim is impaired, such as in galactosemia.

A point of interest in relation to galactosamine 1-phosphate is its resistance to acid hydrolysis. Such a resistance has been observed in glucosaminides [see Ref. (21) for a discussion of this].

Summary

The transfer of phosphate from adenosine triphosphate to galactosamine was found to be catalyzed by a liver enzyme. On the basis of the parallel distribution and from crossed inhibition experiments it is suggested that enzyme may be galactokinase.

The optimum conditions for activity and a method for partial purification are described.

Phosphorylation of galactosamine and of galactose was also found to be catalyzed by

extracts from brain tissue and from a lactose yeast (*Saccharomyces fragilis*). Extracts from cells of the latter grown lactose, which cells contain more galactokinase, were found to have higher activity on galactosamine.

Evidence is presented indicating that the reaction products is galactosamine 1-phosphate. This product was found to be more resistant to acid hydrolysis than aldose 1-phosphates.

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JOURNAL BIOLOGICAL CHEMISTRY
Vol. 203, p. 1055, 1953

URIDINE DIPHOSPHATE ACETYLGLUCOSAMINE *

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Studies on partially purified preparations of uridine diphosphate glucose (1, 2) obtained from yeast revealed the presence of a similar compound containing a different sugar moiety (3). In the course of a systematic study on yeast nucleotides this substance, previously referred to as UDPX and now as UDPAG [1], was obtained in larger amounts. The properties of the sugar moiety are those of acetylglucosamine, and therefore UDPAG is closely related to the compounds found by Park (4) in the cells of *Staphylococcus aureus* treated with penicillin, which contain uridine-5'-pyrophosphate combined with an unidentified amino sugar derivative.

Separation of Yeast Nucleotides

A nucleotide mixture obtained from yeast by extraction with 50 per cent ethanol, followed by precipitation with mercuric chloride and treatment with hydrogen sulfide, was run through an anion exchange resin (Dowex 1) and eluted with solutions of decreasing pH and increasing chloride concentration, following the procedure deschibed by Cohn [5]. The fractions corresponding to each peak were passed through small charcoal columns and the substances were subsequently eluted with ethanol-ammonia. The concentrated solutions thus obtained were analyzed so that the substances corresponding to each peak could be tentatively identified. The results appear in Fig. 1 and Table I.

It is interesting that several uridine compounds were found. One of them could not be identified and the others were UMP-5', UDPG, and UDPAG. In view of the relatively large amount of UDPAG which could be obtained free from UDPG, most of the efforts were concentrated on the former substance. For this purpose a simplified scheme of elution from the resin was employed and, after adsorption on charcoal and evaporation under reduced pressure, the substance was fractionally precipitated as the calcium salt with ethanol.

The amount of UDPAG obtained from fresh bakers' yeast was variable about 200 micromoles per kilo in one experiment and 50 μM in the others. The ratio UDPAG to UDPG in the extract before purification by anion exchange was about 2, except for some samples of toluene-treated yeast (1, 2) in wich it was 0.3 or lower.

Identification of Sugar Moiety

UDPAG liberates by mild acid hydrolysis a substance with about half the reducing power of glucose (assuming 1 molecule of the substance per molecule of uridine) (3).

This substance was found to give a positive reaction for acetylhexosamines, and therefore the statement contained in a previous paper (3) on the negative Elson and Morgan reaction was erroneous. The absorption spectra of the sugar moiety of UDPAG and ace-

* This investigation was supported in part by a research grant (G-3442) from the National Institutes of Health, United Sates Public Health Service, and by the Rockefeller Foundation.

A preliminary report has been published (*Ciencia e Investigación (Buenos Aires)*, 8, 469 (1952).

[1] The following abbreviations will be used: UDPG for uridine diphosphate glucose; UDPAG for uridine disphophate acetylglucosamine. UDP for uridine disphophate, UMP for uridine monophosphate, ADP for adenosinediphosphate, AMP for adenosinemonophosphate, DPN for diphosphopyridine nucleotide.

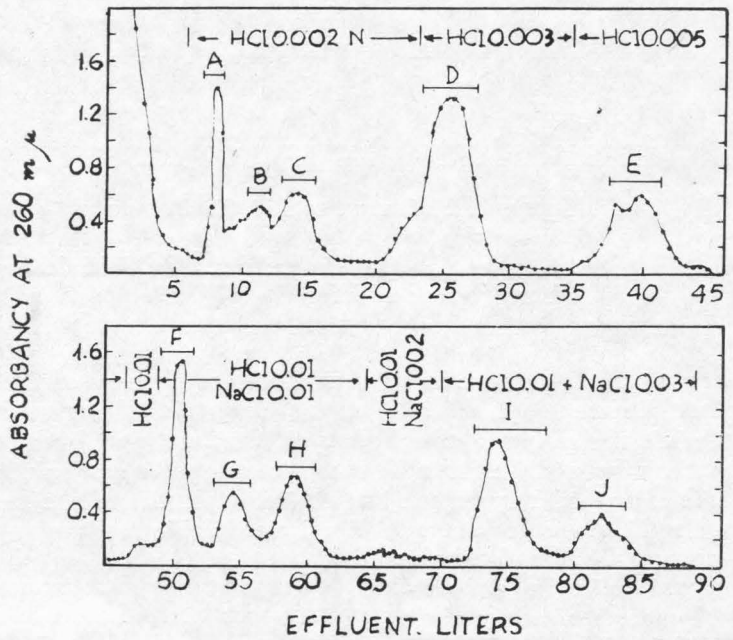


FIG. 1. — Separation of yeast nucleotides by anion exchange. The lines above the peaks represent the fractions of each peak pooled for analysis. Peak B, mainly DPN; Peak C, a uridine derivative plus unknown substance; Peak D, AMP-5'; Peak E, UMP-5' plus unidentified compound; Peak F, inosinic and guanylic acids; Peak G an ADP-ribose compound; Peak H, ADP; Peak I, UDPAG; Peak J, UDPG.

TABLE I

Analytical Data on Effluent Fractions

Substance	Spectrum type (1)	P-nucleoside ratio (2)	Labile P-total P ratio (3)	$R_{adenosine}$ with ethanol-ammonium acetate, pH 7.5 (4)	$R_{adenosine}$ with ethanol-ammonium acetate pH 3.8 (5)	R_F with butanol-disodium phosphate (6)	$R_{adenosine}$ of nucleosides with ethanol-ammonium acetate, pH 3.8 (5)	R_F of bases with ammonium sulfate-Isopropanol**
B	DPN	2.1	0.17	0.37	0.24			
C	Uridine (atypical)	0.71	0.27	0.74	0.60, 0.73	0.90	1.18	
D	Adenosine	1.03	0.06	0.47	0.64	0.75	0.95	0.17
E	Uridine	0.92	0.12	0.55, 0.79	0.62, 0.80	0.88	1.17	
F	Inosine (atypical)	0.98	0.13	0.39	0.51, 0.62		0.95	
G	Adenosine	2.0	0.16	0.61	0.42		0.95	0.17
H	,,	1.75	0.44	0.31	0.36	0.78	0.96	0.17
I	Uridine	1.8	0.49	0.79	0.61		1.18	
J	,,	1.6	0.46	0.68	0.47			

1 Determined in neutral, acid (0.1 N), and alkaline (0.1 N) solutions.

2 Calculated from the total phosphate and the extinction coefficients of the substances as found in the literature.

3 Labile phosphate is defined as the phosphate liberated by 15 minutes hydrolysis in 1 N acid at 100°.

4 $R_{adenosine}$ of adenosine-5'-phosphate, 0.47; inosine-5'-phosphate, 0.41; DPN, 0.38. For other data, see Paladini and Leloir (3).

5 $R_{adenosine}$ of adenosine-5'-phosphate, 0.63; inosine-5'-phosphate, 0.63; DPN, 0.24; uridine-5'-phosphate, 0.79; uridine, 1.16; inosine, 1.02; AMP-3' after phosphatase, 0.95. For other data, see Paladini and Leloir (3).

5 R_F of adenosine-5'-phosphate, 0.75; adenylic acid a, 0.73; adenylic acid b, 0.64.

** R_F of adenine, 0.17.

tylglucosamine after treatment with dilute alkali and *p*-dimethylaminobenzaldehyde, as described by Aminoff *et al.* (6), were compared and found to be identical (Fig. 2).

By paper chromatography of the free sugar with pyridine-ehtyl acetatewater (7), followed by spraying with aniline phthalate (8) or with the modified Elson and Morgan reagent (9), a single spot was obtained, with the same $R_{glucose}$ value and color as acetylglucosamine (Table II). More than twenty sugars have been tested with this solvent. Of these, only acetylgalactosamine and 3-methylgalactose migrated on the paper at a rate similar to that of acetylglucosamine. However, acetylgalactosamine can be easily differentiated from acetylglucosamine by using the same solvent, but with borate-treated papers (Table II), while 3-methylgalactose gives different color reactions.

Identical $R_{glucose}$ values for acetylglucosamine and the sugar from UDPAG were also obtained by chromatography with butanol-ammonia.

When the sugar was submitted to prolonged hydrolysis under conditions which lead to deacetylation of acetylglucosamine, it was found to give a positive Dische and Borenfreund reaction for glucosamine (10). The hydrolysate was run on paper with a solvent mixture composed of ethyl acetate, pyridine, ammonia, and water. The chromatogram, sprayed with the modified Elson and Morgan reagent, showed a residual spot with the same $R_{glucose}$ as acetylglucosamine, and a new one with the same $R_{glucose}$ as glucosamine, as can be seen in Table III.

The isolation of acetylglucosamine was not attempted on account of the relatively small amounts of UDPAG available, but the evidence outlined above leaves little doubt about its identity. As unhydrolyzed UDPAG is non-reducing and gives no color with the Morgan and Elson reagent (11), it may be concluded that acetylglucosamine is linked to the rest of the molecule through the carbon atom 1. The sugar is very easily liberated by dilute acid, as shown in Fig. 3.

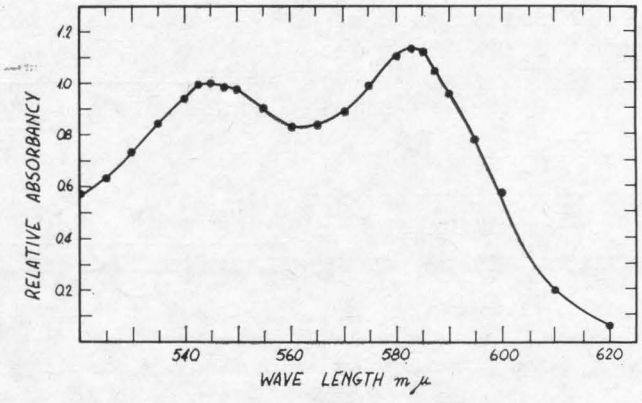


FIG. 2. - Absorption spectra of the sugar moiety of UDPAG (•) and of ecetylglucosamine (solid line) treated with alkali and *p*-dimethylaminobenzaldehyde according to Aminoff *et al.* (6). The absorbancy values were multiplied by a factor so as to make the absorbancy at 545 mμ equal to 1.0.

TABLE II

Paper Chromatography of Sugar Moiety from UDPAG

Solvent, ethyl acetate-pyridine-water (7, 21).

Substance	*R*glucose values of spots	
	Untreated paper	Borate-buffered paper
Acetylglucosamine	1.24	2.25
Acetylgalactosamine	1.14	1.7
Hydrolyzed UDPAG	1.23	2.24

TABLE III

Paper Chromatography of Deacetylated Sugar from UDPAG

Solvent, ethyl acetate-pyridine-ammonia water (see the text).

Substance	*R*glucose values of spots
Acetylglucosamine	1.20
Glucosamine	0.68
Galactosamine	0.53
Deacetylated acetylglucosamine ..	0.69, 1.20 *
„ sugar from UDPAG	0.70, 1.20 *

* Very feeble.

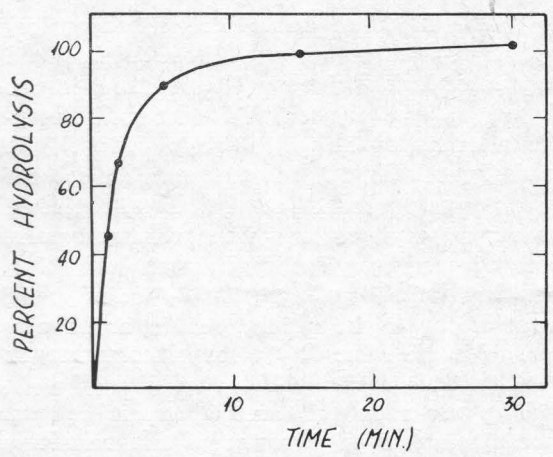


FIG. 3. — Liberation of acetylglucosamine from UDPAG heated in 0.01 N acid at 100°. The value obtained after 30 minutes was taken as 100.

Presence of Uridine

The absorption spectrum of UDPAG was found to be identical with that of uridine (2) and to show the same changes with pH and upon the addition of bromine. Uridine could be identified also chromatographically as a breakdown product of UDPAG (see below).

Presence of Phosphate

As in UDPG, two phosphate groups per molecule of uridine were found. One of them is acid-labile and can be hydrolyzed in 20 minutes with 1 N acid at 100°. Comparative hydrolysis data for UDPAG and UDPG are shown in Table IV.

Acid Hydrolysis of UDPAG

Uridine-5'-pyrophosphate and uridine-5'-monophosphate were identified as breakdown products of UDPAG, after acid hydrolysis, by paper chromatography in two different solvents (Table V). The standards of comparison were a synthetic specimen of UMP-5' and UDP obtained from UDPG. This UDP has been shown by Anand *et al.* (12) to be identical with synthetic uridine-5'-pyrophosphate.

TABLE IV

Phosphate Liberated from UDPAG and UDPG by Acid Hydrolysis

The figures represent the per cent phosphate liberated at 100°, the 30 minute value in 1 N acid being taken as 100.

Time	1 N acid		0.1 N acid	
	UDPAG	UDPG	UDPAG	UDPAG
min.				
15	94	90.7	38.4	41
30	100	100	63	60.7
60	100	100	85	81.3
120			99	94.3

TABLE V

Paper Chromatography of Nucleotides Obtained by Acid Hydrolysis of UDPAG

Substance	Radenosine values of ultraviolet-absorbing spots	
	Ethanol-ammonium acetate, pH 7.5	Ethanol-ammonium acetate, pH 3.8
UDPG	0.43	0.45
UDPAG	0.58	0.60
UDP from UDPG	0.14	0.40
UDPAG heated 10 min. in 0.01 N acid at 100°	0.14	0.39
Synthetic UMP-5'	0.30	0.76
UDPAG heated 20 min. in 1 N acid at 100°	0.30	0.77
UMP-3'	0.40	0.88

The position of the phosphate group in uridylic acid was further confirmed by treatment with a 5'-nucleotidase from snake venom (13). As can be seen in Table VI, the uridylic acid obtained from UDPAG was hydrolyzed by this enzyme yielding inorganic phosphate and a substance with the same $R_{adenosine}$ as uridine, whereas UMP-3' was not attacked.

Alkaline Hydrolysis

It was previously observed (3) that chromatography of UDPG and UDPAG mixtures with an alkaline solvent led to a decomposition of the former substance but to no observable change of the latter. The alkaline decomposition of UDPG was found to give rise to UMP-5' and a cyclic phosphoric ester of glucose. No chromatographically detectable change has been observed after heating UDPAG during 5 minutes at 100° in concentrated ammonia. After heating 15 minutes at 100° in 0.15 N barium hydroxide, two substances could be detected by paper chromatography which appeared to be UMP-5' and acetylglucosamine-1-phosphate (Table VII). The latter substance has been prepared synthetically and will be dealt with in future papers. Park (4) has commented on the different stability to alkali of UDPG and the UDP-amino sugar compounds. The cause of the greater stability would be that in UDPAG the hydroxyl at position 2 is unavailable for the formation of the cyclic phosphoric ester.

Linkage of Different Components

The analytical data for the calcium salt of UDPAG dried over phosphorus pentoxide are shown in Table VIII. The results correspond to a preparation of about 90 per cent purity of a compound containing one uridine, two phosphate groups (one of them being acid-labile), and one acetylglucosamine residue.

The fact that the intact compound is nonreducing and gives no Morgan and Elson reaction and the liberation of UDP and UMP-5' by acid hydrolysis suggest a structure similar to that of UDPG. This structure is further supported by the results of the alkaline hydrolysis.

TABLE VI

Action of 5'-Nucleotidase on Uridine Monophosphate from UDPAG

Each tube contained 0.1 ml. of *Crotalus adamanteus* enzyme, 5 μM of $MgCl_2$, 50 μM of glycine, pH 8.5, 1 μM of uridine monophosphate from UDPAG or UMP-3'. Total volume, 0.4 ml. The mixture was incubated at 37° and the reaction stopped by addition of 0.8 ml. of ethanol. After centrifuging, 0.5 ml. aliquots of the supernatant were taken for inorganic phosphate determinations and the rest was evaporated *in vacuo* and submitted to paper chromatography with ethanol-ammonium acetate, pH 3.8. The $R_{adenosine}$ of synthetic UMP-5' and of uridine were 0.79 and 1.18 respectively.

Substrate added	Time of incubation	Inorganic phosphate in total sample	Radenosine of ultraviolet absorbing substances
	min.	μM	
UMP from UDPAG	0	0.18	0.79
UMP from UDPAG	30	0.95	0.78,* 1.17
UMP-3'	0	0	0.89
UMP-3'	30	0	0.87

* Feeble.

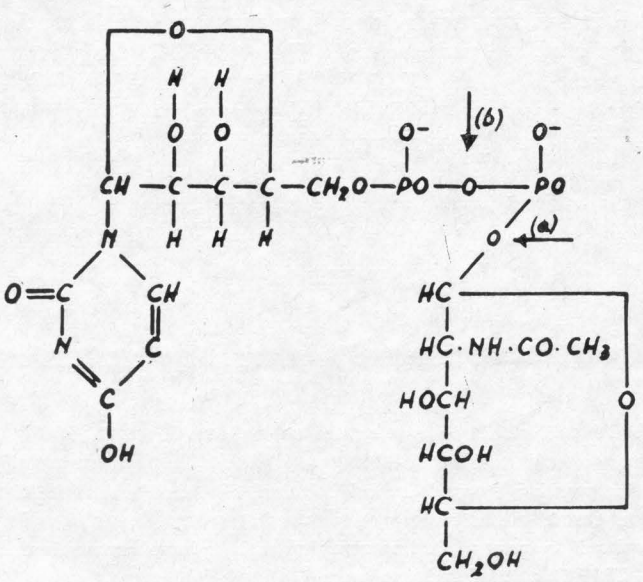
TABLE VII

Paper Chromatography of Alkaline Degradation Products of UDPAG

After hydrolysis, the samples were neutralized with sulfuric acid and centrifuged. The supernatants were passed through a cation exchange resin (Dowex 50). The eluates were neutralized with ammonia evaporated *in vacuo*, and used for chromatography. Solvent, ethanol-ammonium acetate, pH 3.8.

Substance	Time of heating 0.15 N Ba(OH)2	Distance of spots from starting line				
		Located with ultraviolet		Located with P reagent		
	min.	cm.	cm.	cm.	cm.	cm.
UDPAG	0	18.6		18.6		
„	5	18.6	22.5	18.6	22.5	28.8
„	15	18.6*	22.8	18.6	22.8	28.7
Acetylglucosamine-1-phosphate	0					29.7
UMP-5'	0		23.7		23.7	

* Faint.



Uridine diphosphate acetylglucosamine

The compound represented by the accompanying formula would show two primary phosphoric acid groups. Hydrolysis at the points marked *a* and *b* would yield one secondary phosphoric acid group in each case. Besides, hydrolysis at *a* and *b* would liberate acetylglucosamine and inorganic phosphate. Therefore, the electrometric data were compared with the values predicted from total phosphate, inorganic phosphate, and free acetyl glucosamine estimations in samples of the substance before and after hydrolysis. The results, which were exactly equivalent to those obtained previously (1, 2) with UDPG, are summarized in Table IX, and agree well with those predicted from the accompanying formula.

TABLE VIII

Analytical Data for Sample of Calcium Salt of UDPAG

Component	Found	Theoretical for Ca salt	Ratio component-uridine
	μM per mg.	μM per mg.	
Uridine (from absorbancy at 260 mμ)	1.48	1.55	1
Total phosphate	2.75	3.1	1.86
Labile „ (20 min. in 1 N acid at 100°)	1.31	1.55	0.885
Acetylglucosamine	1.49	1.55	1.01
Nitrogen	4.59	4.66	3.12

TABLE IX

Electrometric Titration of UDPAG

The technique was the same as described previously (2).

Time of heating at 100°; about pH 2	Base, μeq			
	Calculated from analytical data		Observed on electrometric titration	
	Primary	Secondary	Primary[1]	Secondary[2]
min.				
0	9.6	0	9.6	0
10	9.6	5.0	9.6	5.2
60	9.6	8.0	9.6	8.2

1 Titrated to pH 4.5.

2 Titrated from pH 4.5 to 8.2.

Park's Compound I differs from UDPAG, since its sugar moiety contains an acid group and does not migrate on paper like acetylglucosamine (4).

Due to the close similarity in the structures of UDPAG and UDPG, and considering the coenzymatic activity of the latter, it may be suspected hat UDPAG plays some rôle in the metabolism of hexosamines. However, preliminary experiments in this direction have been negative.

Experimental

Methods. — Besides those employed in previous paper (1-3), the following analytical methods were used: those of Morgan and Elson (11) or Aminoff *et al.* (6) for acetylglucosamine, Dische and Borenfreund (10) for glucosamine, Johnson (14) for nitrogen, Albaum and Umbreit (15) for pentoses, Kalckar (16) for AMP-5' deaminase, Schlenk and Schlenk for AMP-5', using muscle enzymes (17), Colowick *et al.* (18) for DPN, and Cori and Green (19) for AMP-5', using phosphorylase b.

Nucleosides were prepared from the nucleotides by treatment with a purified pig's kidney alkaline phosphatase (20).

The nucleotides were hydrolyzed to the free bases by heating at 100° in 1 N sulfuric acid during 30 minutes for the purines, and in 2.5 N sulfuric acid during 6 hours for the pyrimidines.

Paper chromatography of sugars was carried out with the ethyl acetatepyridine-water mixture previously used (7, 21). Acetylglucosamine and acetylgalactosamine were separated by using papers which had been immersed in 0.2 M borate buffer of pH 8 and dried. Glucosamine and galactosamine showed a marked tailing with the above mixture, but gave well defined and separated spots after running with a more alkaline solvent prepared by mixing ethyl acetate, pyridine, concentrated ammonia, and water in the proportions 10:5:3:3 by volume. After equilibrating at 30°, the upper phase was used.

Sugars were located as described by Partridge with aniline phthalate (8) or with a modified Elson and Morgan reagent (9).

For paper chromatography of nucleotides and nucleosides, the ethanol-ammonium acetate mixtures already described (3) were used, in addition to Carter's isoamyl alcohol-disodium phosphate solvent (22). The chromatography of free purine and pyrimidine bases was carried out with isopropanol-ammonium sulfate (23) or with butanol-ammonia (24). Standard substances were run in every chromatogram, since no precautions were taken to obtain reproducible R_f values.

The position of ultraviolet-absorbing substances was ascertained with a Mineralight lamp and that of phosphorylated compounds according to Bandurski and Axelrod (25). The position of the substances on the paper is given relative to the position of glucose or adenosine. For instance, the ratio of the distance traveled by the unknown substance to the distance traveled by glucose is referred to as $R_{glucose}$

Yeast Extract. — To 10 kilos of bakers' yeast, 10 liters of 95 per cent ethanol were added and the mixture was heated with continuous stirring until it boiled. On the following day it was filtered through a 32 cm. Büchner funnel with a filter-aid. The filtrate was acidified with 5 N nitric acid until acid to Congo red paper. Then 30 ml. of mercuric acetate (2) per liter were added. After mixing, the preparation was left overnight in the refrigerator. The suspension was filtered through a Büchner funnel. The precipitate was dried as much as possible by suction, and placed in a blendor with 1200 ml. of water, and decomposed with hydrogen sulfide in the cold. The mercuric sulfide was filtered off and washed with 100 ml. of water, and the combined filtrates were aerated and neutralized to pH 6. Prior to chromatography, the solution was brought to pH 7.5 by addition of concentrated ammonia.

Column Chromatography. — A glass colum (50 cm. high and 4.5 cm. inner diameter) was fed through a rubber tubing by a container hung about 1.5 meters above.

The fraction collector consisted of a row of forty flat bottles of 1 liter capacity over which the column was displaced on rails by an electrically driven motor, so that a distance of about 2 meters was covered in 24 hours. The tip of the column was fitted with a hanging funnel and an escape mechanism controlled by a row of suitably spaced nails which prevented the loss of effluent in the space between the bottles.

The strong base Dowex 1 anionic resin was employed throughout. The resin (200 to 400 mesh) was converted to the chloride form with 1 N hydrochloric acid and freed from fines by six or more decantations from water.

A smaller amount of coarser resin, obtained by further decantations and suspended in water, was poured into the column to form a layer 2 to 3 cm. thick, which prevented leakage of the smallest particles through the fritted glass disk. The rest of the resin, suspended in a small amount of water, was then added and allowed to settle. The column, which was 30 cm. high, was washed with 1 N hydrochloric acid until the absorbancy of the effluent dropped to a value of 0.03 to 0.04, followed by water until the pH of the effluent was about 5.

The solution of nucleotides (1500 ml., containing about 8000 μM calculated as uridine from the absorbancy at 260 mμ) was allowed to drain at a rate of 6 to 8 ml. per minute. From this point on, the procedure varied in different cases.

(*a*) For the type of experiment shown in Fig. 1, the column was first washed with water until the absorbancy of the effluent dropped below 0.1. Then the first eluent (0.002 N hydrochloric acid) was run through and the elution was followed by measurements of the absorbancy at 260 mμ. The rate of flow was maintained between 6 and 8 ml. per minute. Each eluent was replaced by the next one after the absorbency of five to ten fractions (2.5 to 5 liters) had remained under 0.1.

The highest concentration of hydrochloric acid used was 0.01 N in order to prevent decomposition of labile nucleotides, and solutions of higher eluting power were prepared by addition of sodium chloride.

(*b*) When it was desired to isolate only UDPAG and UDPG, a simplified procedure was employed. After a washing with water (500 ml.), the following solutions were successively run through the column: 0.01 N hydrochloric acid 0.01 N sodium chloride in 0.01 N hydrochloric acid, 0.02 N sodium chloride in 0.01 N hydrochloric acid.

Each eluent was replaced by the next, the same criterion as in (*a*) being used. Finally, UDPAG and UDPG could be eluted separately with 0.03 N sodium chloride in 0.01 N hydrochloric acid. In extracts from toluene-treated yeast, which contained less UDPAG than UDPG, a complete separation between the two substances could be obtained only when a solution of 0.025 N sodium chloride in 0.01 N hydrochloric acid was used in the last step.

After each run the column was regenerated with 1 N hydrochloric acid, followed by water. The same column was used several times, except for a small layer at the top which darkened during the run and was replaced each time by fresh resin.

Concentration. — The method of Cohn (5), which makes use of small resin columns, was discarded in view of the conditions of high acidity to which the substances are exposed during the process of concentration. Instead an alternative procedure was devised which yielded excellent results in most cases. The fractions belonging to each peak were pooled and absorbed on a small charcoal column (3 gm. of Norit A in a fritted glass funnel 4 cm. in diameter). Elution from the charcoal was carried out with a water-ethanol-ammonia mixture (40 ml. of 95 per cent alcohol plus 1 ml. of concentrated ammonia, made up to 100 ml. with water).

Each fraction of 3 to 5 ml. was collected in the cold and immediately adjusted to pH 5 to 6 with hydrochloric acid. The fractions of high absorbancy were then pooled. It was thus possible to concentrate the solutions from several liters to 20 to 40 ml. The yield of this step was 70 to 80 per cent.

These solutions were further concentrated by evaporation under reduced pressure, and in some cases the nucleotides were precipitated as the calcium salt by addition of some drops of a saturated solution of calcium chloride in ethanol, followed by several volumes of ethanol until no more precipitation occurred.

Analysis of Effluent Fractions. — Some of the results obtained from the study of the fractions isolated in the experiment of Fig. 1 are summarized in Table I. Besides the analytical data obtained for each compound, further information was provided by the composition of the eluent, since a lower pH and a higher anion concentration are needed for the elution of the ions with higher net negative charge (5).

A considerable amount of ultraviolet-absorbing material, representing about 30 per cent of the total absorbancy of the sample, was not adsorbed on the column and was recovered in the wash water. This fraction, which contained no phosphate, probably consisted mainly of nucleosides and free bases. On standing, it gave rise to a crystalline precipitate which seemed to be hypoxanthine, as judged by its spectrum.

Peak A was not analyzed, as most of it was lost in the process of concentration.

Peak B gave an adenosine spectrum. A band at 340 mμ appeared in cyanide solution. The R_F values were those of DPN. It catalyzed the oxidation of alcohol and glyceraldehyde phosphate in the presence of yeast enzymes and dichlorophenol indophenol. The material contained in Peak, B therefore appeared to be DPN, but some contaminant was present since the phosphate content was too high for the DPN values calculated from the absorbancy at 340 mμ.

Peak C. — Two spots were obtained by paper chromatography with one of the solvents. After separation of the two substances, one (C_1) was found to give a spectrum similar to that of uridine, while the other (C_2) gave a spectrum which could not be identified (maximum 260 mμ in acid, 230 and 260 mμ in alkali).

The R_F of the nucleoside prepared from C_1 corresponded to that of uridine. Acid hydrolysis gave a substance of spectrum and R_F corresponding to uracil. In addition, two other substances appeared, one probably being UMP while the other could not be identified. Therefore, Peak C contained two substances, one of which is a uridine compound.

Peak D — The data obtained for this substance were as follows. The spectrum was typical for adenosine. There was one phosphate for each adenosine, 6 per cent of which was acid-labile. The R_F values in three solvents corresponded to AMP-5', and the substance obtained after hydrolysis with phosphatase gave the R_F value of adenosine and that of adenine after acid hydrolysis.

The substance stimulated phosphorylase b and the dephosphorylation of phosphopyruvate as described by Schlenk and Schlenk (17). Muscle deaminase produced the same spectral changes as on AMP-5'. Fron this evidence it can be concluded that the substance in Peak D is AMP-5'.

Peak E gave two spots on paper chromatography, one of them (E_2) corresponding to

UMP-5', as judged by the R_F value and by the type of the spectrum. The other compound (E_1) gave a spectrum very similar to that of C_2. The results obtained after phosphatase or acid treatment of Peak E were very similar to those for Peak C. Therefore, Peak E appeared to contain UMP-5' and an unidentified compound.

Peak F — The spectrum was somewhat similar to that of inosine. Analysis showed one phosphate per nucleoside residue. Two spots appeared on chromatography with one solvent and the R_F of one of these spots corresponded to that of inosinic acid. After elution of the substances in each spot, the faster gave a typical inosine spectrum and the slower a guanosine type of spectrum. The bases liberated by acid hydrolysis were hypoxanthine and guanine, as judged by paper chromatography, followed by elution and spectrophotometry.

Thus Peak F appeared to contain a mixture of inosinic and guanylic acids. Considering the acid stability of the phosphate, these substances probably corresponded to the 5' isomers.

Peak G — A typical adenosine spectrum was obtained. No spectral change was observed after addition of cyanide. Two phosphate groups per adenosine were found. Paper chromatography of the nucleoside and base gave R_F values equal to those of adenosine and adenine respectively. Two ribose residues per each adenosine were found. The same data would be obtained for the product resulting from hydrolytic removal of the nicotinamide from DPN.

Peak H gave a typical spectrum for adenosine. The phosphate-adenosine ratio was 1.75, and 44 per cent of the phosphate was labile. Adenosine and adenine were obtained by hydrolysis. In the presence of crude hexokinase, phosphate was transferred to glucose. The bulk of the substance was probably ADP.

Peak I — The substance contained in the fraction is referred to as UDPAG and was studied in detail.

Peak J — The data shown in Table I, the chromatographic behavior and the coenzymic activity corresponded to those of UDPG.

Degradation Products of UDPAG

Identification of Acetylglucosamine — For the quantitative tests, UDPAG was hydrolyzed with 0.1 N sulfuric acid during 10 minutes at 100°, the mixture was neutralized with 0.3 N barium hydroxide, and the nucleotides precipitated by adding equal volumes of 5 per cent zinc sulfate and 0.3 N barium hydroxide. After centrifuging, acetylglucosamine was determined according to Aminoff *et al.* (6) in an aliquot of the supernatant solution.

To obtain a solution of the sugar suitable for chromatography, UDPAG was hydrolyzed as above and the liquid was treated successively with a cation exchange and an anion exchange resin (Dowex 50 and Amberlite IR-4B). This solution was evaporated *in vacuo* and submitted to paper chromatography (Table II).

The deacetylation of the sugar was carried out on an aliquot of the same solution, which was heated in a sealed tube with 0.1 N sulfuric acid 24 hours at 100°. The liquid was neutralized with barium hydroxide, centrifuged, and the supernatant solution used for chromatography. A control with an authentic sample of acetylglucosamine was run simultaneously (Table III).

Uridine-Diphosphate — UDPAG was hydrolyzed with 0.01 N sulfuric acid during 10 minutes at 100°. All the sugar but practically no phosphate is liberated under these conditions. The acid was neutralized with dilute ammonia and the solution used for paper chromatography. For comparison, a sample of UDPG was submitted to the same treatment (Table V).

Uridine Monophosphate — UDPAG was hydrolyzed in 1 N sulfuric acid during 20 minutes at 100°. The acid was neutralized with barium hydroxide, and the supernatant solution used for chromatography (Table V).

Action of 5'-Nucleotidase on Uridine Monophosphate — For this experiment uridine monophosphate was prepared from UDPAG as described above, except that an excess of barium hydroxide (to pH 8 to 9) was added in order to precipitate most of the inorganic phosphate. After centrifuging, the supernatant fluid was neutralized with sulfuric acid

and centrifuged again. The resulting solution was incubated with the enzyme, as described by Heppel and Hilmoe (13) (Table IV).

The authors wish to express their gratitude to Professor A. R. Todd for a sample of synthetic uridine-5'-phosphate, to Dr. L. A. Heppel for the *Crotalus adamanteus* enzyme ,and to Dr. R. Caputto, Dr. J. L. Reissig, and Dr. R. E. Trucco for helpful criticism.

SUMMARY

A preparation of nucleotides from bakers' yeast has been fractionated by chromatography on an anion exchange resin. The compounds contained in each fraction were tentatively identified and one of them was studied in more detail. This substance (UDPAG) gives the same spectrum as uridine at several pH values and after treatment with bromine. It contains two phosphate groups, one of which is acid-labile.

Mild acid hydrolysis liberates a substance identical with acetylglucosamine, as judged by color reactions and paper chromatography with different solvents. Moreover, by further acid treatment of the sugar moiety, a compound is released which shows the same chemical properties and chromatographic behavior as glucosamine.

The nucleotides set free from UDPAG by acid hydrolysis migrate on paper like the UDP and UMP obtained from UDPG.

Alkaline hydrolysis liberates substances behaving like UMP-5' and acetylglucosamine-1-phosphate.

These facts, together with the analytical data and the results of the electrometric titration, lend support to a structure for UDPAG in which uridine-5'-pyrophosphate and acetyl glucosamine are joined through a glycosidic link.

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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Vol. 75, p. 5445, 1953

THE ENZYMIC SYNTHESIS OF TREHALOSE PHOSPHATE [1]

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Uridine diphosphate glucose (UDPG) [2] has been found to disappear when incubated with a yeast extract and glucose monophosphate. This disappearance may be measured by estimating UDPG by its coenzymatic activity [3] and also as a decrease in acid-labile glucose. During the reaction UDP is formed and the reducing power of the mixture decreases. As shown in Table I, these changes are equivalent and do not take place when any one of the reactants is added at the end of the incubation period.

TABLE I

Analytical Changes Produced by the Enzyme

Incubation of 0.4 μmole of glucose-6-phosphate, 0.6 μmole of UDPG and 0.02 ml. of enzyme in 0.14 *M*/tris (hydrosymethyl) -aminomethane buffer of *p*H 7 during 100 minutes at 37°; total volume, 0.1 ml.; results expressed in μmoles. The enzyme was obtained by disintegrating brewer's yeast cells with sand in a 50 cycles per second oscillator. After centrifuging the supernatant was made 0.5 saturated with ammonium sulfate and the precipitate was dialyzed.

Sample	Substance omitted during incubation *a*	Δ Reducing Power *b*	Δ Labile Glucose *c*	Δ UDP *d*
1	Glucose-6-phosphate	0	−0.04	+0.02
2	UDPG	0	0	0
3	None	−0.13	−0.14	+0.14

a The substance omitted was added at the end of the incubation period. The Δ values represent the difference with sample 2. *b* Calculated as glucose. *c* Hydrolyzed 10 minutes at pH 2 followed by precipitation with zinc sulfate and barium hydroxide. Practically all the glucose liberated under these conditions is that of UDPG. *d* Estimated by a method based on the reacton: phosphopyruvate + UDP → pyruvate + UTP (A. Kornberg, in "Phosphorus Metabolism". The Johns Hopkins Press, Baltimore, Md., 1951, Vol. 1, p. 392). Pyruvate measured colorimetrically.

Samples equal to those shown in Table I were submitted to fractionation of the barium salts. The water soluble, alcohol-insoluble fractions were used for paper electrophoresis with borate buffer [4] and the phosphate containing compounds were subsequently developed with a molybdate spray reagent [5]. The experiment showed that sample 3, but not samples 1 or 2, contained a phosphate compound which migrated at 60 per cent the rate of glucose-6-phosphate. De phosphorylation of this compound with kidney phosphatase produced a substance which gave the same R_f value as trehalose when chromatographed on paper.

In other experiments the reaction products were deproteinized by heating, treated with charcoal in order to remove the nucleotides and submitted to the action of phosphatase. When chromatographed on paper a substance migrating like trehalose was found to be present in sample 3 but not in the others. The substance extracted from the paper was hydrolyed in 1 *N* acid during 3 hours at 100° and compared chromatographically with trehalose treated in the same manner. In both cases a glucose and a trehalose spot were obtained.

1) This investigation was supported in part by a research grant (G-3442) from the National Institutes of Health, U. S. Public Health Service an by the Rockefeller Foundation.

(2) These abbreviations will be used: UDPG for uridine diphosphate glucose, UDP for urldine disphosphate, and UTP for uridine triphosphate.

(3) R. Caputto, L. F. Leloir, C. F. Cardini and A. C. Paladini. *Biol. Chem.*, 184, 333 1950).

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The solvent used for paper chromatography was pyridine ethyl acetate water[6] with which trehalose, saccharose, maltose and lactose can be separated and the developer was an alkaline silver reagent[7] which reacts slowly with non-reducing disaccharides. Furthermore, reducing from non-reducing sugars can be distinguished because only the latter give color with the aniline-phthalate spray reagent[8]. Thus the ester appears to be a phosphate of trehalose which is presumably identical to that isolated by Robinson and Morgan[9] from the products of yeast fermentation.

The enzyme has been only partially purified and still contains the enzymes which transform glucose-6-phosphate into glucose-1-phosphate and into fructose-6-phosphate, but the most simple explanation of the chemical changes observed is the equation.

UDPG + glucose-6-phosphte →
UDP + trehalose pnosphate

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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Vol. 75, p. 6084, 1953

THE BIOSYNTHESIS OF SUCROSE [1]

BY L. F. LELOIR AND C. E. CARDINI

A previous note [2] reported the formation of trehalose phosphate from UDPG [3] and glucose-6-phosphate. Following the same general procedure, an enzyme has now been found in wheat germ which catalyzes the reaction UDPG + fructose $\rightleftarrows$ sucrose + UDP. The evidence is as follows. The product formed was found to be non-reducing and to behave like sucrose on paper chromatography with two solvents (butanol-acetic acid) [4] and ethyl acetate-pyridine [5]. After extraction of the substance from the paper followed by hydrolysis with dilute acid (5 minutes at *p*H 2 at 100°) or with purified invertase, glucose and fructose were detected chromatographically.

As shown in Table I, equal amounts of sucrose and UDP are formed in the reaction. The disappearance of UDPG and the formation of UDP were checked semiquantitatively after separation by paper chromatography with ethanol-ammonium acetate-Versene [6] as solvent.

The same chromatographic procedure was used for studying the reversibility. Starting with UDP and sucrose it was found that UDPG is formed. Its identity was checked by extracting it from the paper and measuring the coenzymic activity on galactowaldenase [8]. The data indicate that the equilibrium is displaced in favor of sucrose synthesis.

No sucrose formation or UDPG disappearance was found to occur if glucose-1-phosphate was added instead of UDPG, or if sorbose, aldose arabinose or the 1-or 6- phosphates of fructuose or glucose were substituted for fructuose.

TABLE I

The complete system contained 0.05 μmole of UDPG, 2 μmoles of fructose and 0.05 ml. of enzyme ,0.1 ml. of 0.1 *M* sodium dicthyl barbiturate: final volume, 0.25 ml.; *p*H 8.6; incubated during 10 minutes at 37°. The γ values represent the difference in μmoles with a non-incubated sample.

	Sucrose *b*	UDP *c*	Inorganic phosphate
Complete system	0.33	0.30	0.05
No UDPG	0	0	0
No fructose	0	0.05	0.14

The enzyme was obtaed by extracting wheat germ with three volumes of phosphate buffer 0.05 *M*, *p*H 7. After centrifrging the supernatant was dialyzed overnight cold and centrifuged again. The supernatant was precipitated twice by adding 35 g. of ammonium sulfate per 100 ml. The precipitate was suspended in water, dialyzed for 2 hours and adjusted to *p*H 5. The precipitate was redissolved in water at *p*H 7. The precipitation with acid was repeated three times. The solution contained 40 mg. of protein per ml. *b* Sucrose was estimated by the resorcinol method [7] after destroying the fructose by heating 10 minutes at 100° in 0.01 *N* NaOH. *c* Determined enzymatically. [2]

1. This investigation was supported in part by a research grant (G-3442) from the National Institutes of Health, United States Public Health Service, and by the Rockefeller Foundation.

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3. The abbreviations UDPG for uridine diphosphate glucose, and UDP for uridine diphosphate are used.

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Although sucrose had been previously obtained by enzymic action, the mechanism of the synthesis in plants remained obscure. The enzyme which Doudoroff and Hassid extracted from *Pseudomonas saccharophyla* catalyzes the formation of sucrose from glucose-1-phosphate and fructose, but it has not been possible to detect such a reaction in plan material [9]. The enzyme described in this paper has been found to be present not only in wheat germ but also in corn and bean germs and in potato sprouts. Tests for UDPG by its coenzymic activity gave positive results on wheat germ extracts.

Moreover, Buchanan, *et al.* [10] have published evidence of the presence of UDPG in other plants They also suggested that it was envolved in sucrose syntesis, probably by reacting with fructose phosphate to give sucrose phosphate. The latter substance can be excluded as an intermediate in the reaction catalyzed by the wheat germ enzyme because the product is all free sucrose and only negligible amounts of inorganic phosphate are released (Table I).

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JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 206, p. 779, 1954

GUANOSINE DIPHOSPHATE MANNOSE *

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Uridine diphosphate glucose (UDPG) [1] has been isolated from yeast and found to act as coenzyme in the transformation of galactose-1-phosphate to glucose-1-phosphate (1,2). Similar compounds in which the glucose residue is replaced by galactose (3), acetylglucosamine (4), an amino sugar derivative plus amino acids (5), or glucuronic acid (6) have been described.

This paper describes the isolation of a related compound which was first detected by paper chromatography of UDPG preparations purifield by anion exchange and which appears to be guanosine diphosphate mannose (GDPM).

Isolation of GDPM—The starting material was a nucleotide mixture obtained from bakers' yeast. The UDPG content has been found to increase by a short period of autolysis (2), and hence in the preparation of UDPAG (4) it was found convenient to omit this treatment in order to obtain a higher ratio UDPAG: UDPG. Better results for GDPM were obtained by including the autolysis step.

The yeast nucleotides were extracted with 50 per cent ethanol, followed by precipitation with mercuric acetate. In the preparation of UDPG (2) this precipitate was separated in two fractions, one soluble and another insoluble in 1 M ammonium acetate. The soluble fraction was found to contain most of the UDPG and UDPAG, while nearly all the GDPM remained in the insoluble fraction.

In the further purification of GDPM the nucleotide mixture obtained by descomposition of the mercury salts with H_2S was fractionated with an anion exchange colum. In some cases GDPM and UDPG were eluted from the column in a single peak, and the two substances had to be separated by paper chromatography. When the UDPG content of the extract was not very high, as in the experiment shown in Fig. 1, a good separation was obtained. The GDPM-containing fractions were concentrated by adsorption and elution on charcoal. The product obtained was contaminated with a substance having the properties of guanosine-5'-phosphate. This compound was probably produced by decomsition of GDPM during the concentration process, since GMP-5' emerged from the column much earlier than GDPM (Fig. 1). The two substances were separated by ionophoresis on starch, after which GDPM was precipitated as the calcium salt.

Nucleotide Moiety—The ultraviolet absorption spectrum of the substance is presented in Fig. 2. The curve is nearly identical to that of guanosine and shows the same changes in acid and in alkaline solution.

Calculations based on phosphate estimations and on the guanosine content obtained from absorbancy values gave two phosphate groups per guanosine residue.

The hydrolysis curves of the phosphate groups are presented in Fig. 3. In 0.1 N acid at 100° 50 per cent of the phosphate was liberated in 120 minutes. There was a break in the curve for 1 N acid at 100° at 50 per cent hydrolysis, and the second part of the

* This investigation was supported in part by a research grant (No. G-3442) from the National Institutes of Health, United States Public Health Service, and by the Rockefeller Foundation.

1 The following abbreviations will be used: UDPG for uridine diphosphate glucose, GDPM for guanosine diphosphate mannose, UDPAG for uridine diphosphate acetylglucosamine, AMP-5' for adenosine-5'-phosphate, ADP for adenosinediphosphate, UMP-5' for uridine-5'-phosphate, UDP for uridine diphosphate, GMP-5' for guanosine-5'-phosphate, and GDP for guanosine diphosphate.

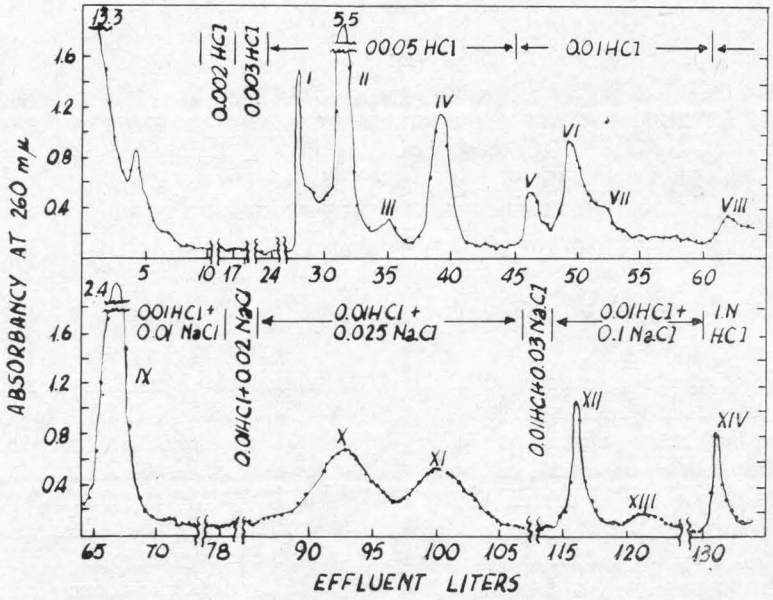

FIG. 1. — Separation of yeast nucleotides by ion exchange (see Preparation 2 under "Methods"). The figures for HCl and NaCl represent the molarity. Probable identity of the substance in each peak: Peak II, AMP-5'; Peak IV, UMP-5'; Peak VI, GMP-5'; Peak VII, inosinic acid; Peak IX, ADP; Peak X, GDPM plus UDPAG; Peak XI, UDPG; Peak XII, UDP plus unidentified compound; Peaks I, III, V, VIII, XIII, and XIV, unidentified substances.

curve was parallel to that of guanosine-5'-phosphate. In the case of UDPG, the rate of hydrolysis of the acid-labile phosphate group is similar to that of GDPM, but the second phosphate group is more stable. This agrees with the known difference in stability of the 5'-phosphates of uridine and guanosine (8).

Sugar Moiety. — Mild acid hydrolysis of GDPM leads to the liberation of a reducing substance. By paper chromatography of this substance with two differents solvent, followed by spraying with aniline phthalate (9) or benzidine reagent (10), a single spot was obtained, of which the position on the paper

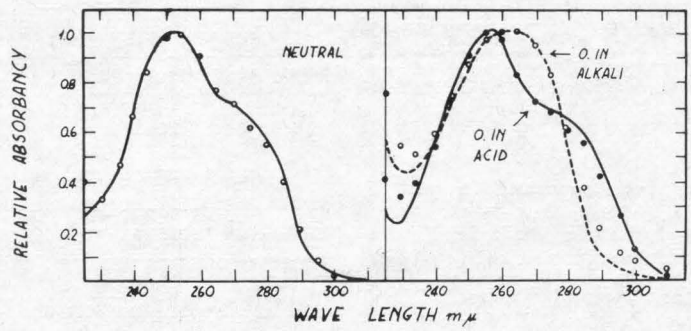

FIG. 2. — Absorption spectra of GDPM and guanosine. The absorbancy value at the maximum was taken as equal to 1. Solid and broken lines, guanosine (from the data of Hotchkiss (7); closed and open circles, GDPM.

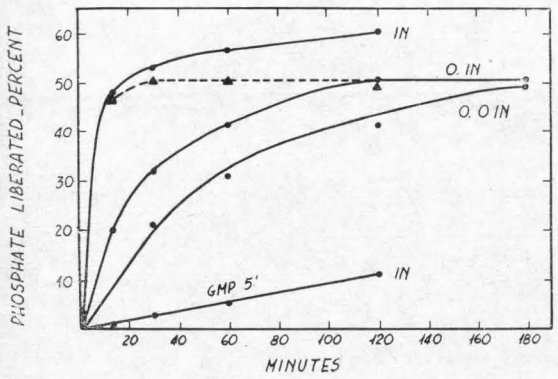

FIG. 3. — Hydrolysis curves for phosphate of GDPM in acid at 100°. The percentages for GMP-5' were divided by 2 in order to obtain a curve directly comparable with that of GDPM. The broken line was obtained by subtracting the values for GMP-5' from those for GDPM in 1 N acid.

and the color were the same as for authentic mannose. In order to confirm the identity of the sugar, independent method, ionophoresis on borate-buffered paper (11), was used. The results are shown in Table I.

Mannose is very easily liberated from GDPM by 0.01 N acid, as can be observed in Fig. 4. As unhydrolyzed GDPM in nonreducing, it may be concluded that mannose is linked to the rest of the molecule through carbon atom 1.

Acid Hydrolysis of GDPM—When GDPM was heated during 15 minutes at 100° at its own acidity (concentration of the solution, 2 μM per ml.; pH about 2.7), all the mannose and very little phosphate (about 10 per cent) were liberated. Thus, guanosine diphosphate was expected to be present in the hydrolysate. Accordingly, chromatograms with two different solvents showed a slow moving, ultraviolet- absorbing spot, which gave a positive reaction with the Hanes and Isherwood spray reagent for phosphate (12) (see Table II). The reaction with the benzidine color reagent for sugars was negative, while it was slightly positive with GDPM.

Hydrolysis of GDPM under the same conditions as above, but during 180 minutes, resulted in the liberation of about half the total phosphate and about 10 per cent of the guanine. The latter precipitated upon neutralization and was identified by its absorption spectrum. The supernatant liquid was submited to paper chromatography with two

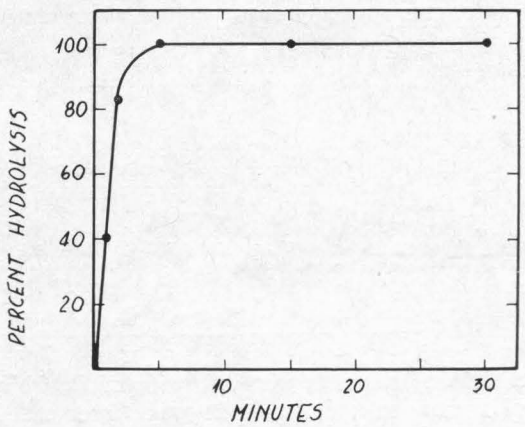

FIG. 4. — Liberation of mannose from GDPM heated in 0.01 N acid at 100°. The value obtained after 15 minutes was taken as 100.

TABLE I

Paper Chromatography and Ionophoresis of Sugar from GDPM

Ionophoresis was carried out on a Whatman No. 1 sheet, 57 cm. long and 9.2 cm wide. A potential of 500 volts was applied during 7 hours.

Substance	Chromatography		Ionophoresis
	*R*xylose with pyridine-ethyl acetate-water	*R*ribose with phenol-ammonia	*R*glucose with borate buffer
Glucose	0.83	0.61	
Galactose	0.71	0.70	0.88
Fructose	0.87	0.83	0.86
Mannose	0.91	0.71	0.64
Sugar from GDPM	0.90	0.71	0.66

TABLE II

Paper Chromatography of GDP and GMP from GDPM

	*R*adenosine values of ultraviolet-absorbing spots	
	Ethanol-ammonium acetate, pH 7.5	Ethanol-ammonium acetate, pH 3.8
GDPM	0.31	0.29
GDPM heated 15 min., pH 2.7, 100°	0.12	0.24
GDPM heated 180 min., pH 2.7, 100°	0.18	0.43
Synthetic GMP-5'	0.19	0.43
Yeast guanylic acid	0.23	0.60

TABLE III

Action of 5'-Nucleotidase on Guanosine Monophosphate from GDPM

The technique was as described by Cabib *et al.*(4). The amount of substrate was 0.24 μM per tube. The R_{uridine} of authentic guanosine was 0.79.

Substrate added	Time of incubation	Inorganic phosphate	*R*uridine of ultraviolet-absorbing substances
	min.	*μM*	
GMP from GDPM	0	0.03	0.22
" " "	30	0.17	0.74
Synthetic GMP-5'	0	0.02	0.21
" "	30	0.19	0.73
Yeast guanylic acid	0	0.03	0.27
" " "	30	0.03	0.27

different solvents. An ultraviolet-absorbing, phosphate-containing spot was observed. Its $R_{\text{adenosine}}$ value was the same as that of synthetic guanosine-5'-phosphate and clearly different from that of yeast guanylic acid (see Table II).

The position of the phosphate group in the guanylic acid was further confirmed by treatment with a specific 5'-nucleotidase from snake venom (13). As can be seen in Table III, both the guanylic acid obtained from GDPM and synthetic GMP-5' were hydrolyzed by the enzyme, yielding inorganic phosphate and a substance with an R_{uridine} value similar to that of guanosine, while yeast guanylic acid was not attacked. The small difference between the R_{uridine} values of authentic guanosine and of the guanosine obtained from the guanylic acids is to be ascribed to the presence of salts in the enzymatic hydrolysates.

Chromatography of the products of hydrolysis of GDPM in 1 N acid during 30 minutes at 100° revealed the presence of a substance which behaved like guanine. The R_F values with an isopropanol-hydrochloric acid solvent (14) were 0.26 for hydrolyzed GDPM, 0.25 for guanine, and 0.39 for adenine.

Structure of GDPM—The analytical data for the calcium salt of GDPM appear in Table IV. The results correspond to a preparation of about 75 per cent purity of a compound containing one guanosine, two phosphate groups (one of them being acid-labile), and one mannose residue.

The fact that the intact compound is nonreducing and that a guanosine diphosphate and GMP-5' are liberated by acid hydrolysis suggets a structure similar to that of UDPAG, as represented in the accompanying formula. Such a compound, after being passed through a cation exchange resin in the acid form, should show on electrometric titration two

TABLE IV

Analytical Data for Sample of Calcium Salt of GDPM

The substance was dried over phosphorus pentoxide at 56° during 4 hours. In order to minimize the error due to non-specific ultraviolet-absorbing substances, the guanosine concentration was calculated from the absorbancy at two wave-lengths in neutral solution as follows. Guanosine concentration (micromoles per ml.) = $(A_{250} - A_{\lambda})\ (A_{m,250} - A_{m,\lambda}) \times 10^3$, where A_{250} represents the absorbancy of the sample at 250 mμ $a_{m,250}$ the molar absorbancy at 250. The corresponding values at another wave-length are reresented as A_{λ} and $A_{m,\lambda}$. With the data of Hotchkiss (7) the same results were obtained by setting λ equal to 230 or 280 mμ.

Component	Found	Theoretical for Ca salt	Moles of component; total phosphate taken as 2.00
	μM per mg	*μM per mg.*	
Guanosine	1.28	1.55	1.08
Total phosphate	2.36	3.1	2.00
Labile phosphate, 120 min. in 0.1 N acid, 100°	1.23	1.55	1.04
Mannose	1.04	1.55	0.88

acid groups, one dissociating as a primary phosphate and the other as the ammonium cation of guanosine (15). Hydrolysis at the points marked *a* and *b* would yield one secondary phosphoric acid group in each case. Besides, hydrolysis at *a* and *b* would liberate mannose and inorganic phosphate respectively. Therefore, the electrometric data were compared with the values predicted from total phosphate, inorganic phosphate, and free mannose estimations in samples of the substance, before and after hydrolysis. The results, summarized in Table V, while not so clear cut as in the paper was usually referred to the position of an appropriate standard (glucose, xylose, or ribose for sugars, and adenosine or uridine for nucleotides); the results are given in the form.

$$R_{\text{standard}} = \frac{\text{distance traveled by unkpown}}{\text{distance traveled by standard substance}}$$

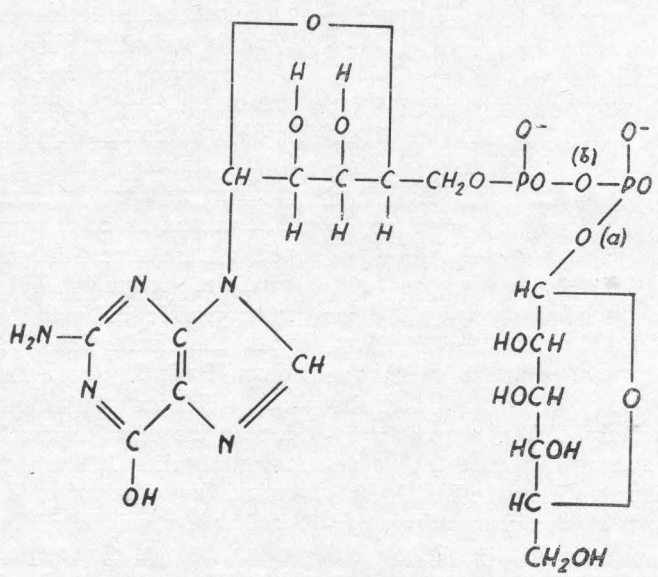

TABLE V

Electrometric Titration of GDPM

The technique was as described previously (2).

Sample	Time of heating at 100°, pH about 2.7	μeq. of base			
		Calculated from analytical data		Observed on electrometric titration	
		Primary	Secondary	Primary *	Secondary [a]
	min.				
A	0	5.37	0	5.84	0 [b]
B	15	6.0	3.51	6.9	3.2
C	180	6.2	5.94	6.4	5.6

* Corresponds to the dissociation of a primary phophate group and the ammonium group of guanosine. Titrated to pH 4.5.

a Titrated from pH 4.5 to 8.2.

b A small amount of alkali (1.3 μeq.) was used to shift the pH from 4.5 to 8.2,but buffering action was at a minimum around the pH zone of secondary phosphate; that is, from pH 6 to 7.5. Threfore, it was assumed that this amount of alkali was used to tirate impurities, and it was subtrated from all the titrations.

Preparation of GDPM

Preparation I—In this case a nucleotide mixture, obtained as for the preparation of UDPAG but from toluene-treated yeast (950 ml. containing about 6000 μM of nucleotides calculated as uridine from the absorbancy at 260 mμ), was submitted to chromatography on the same Dowex 1 column previously employed for UDPAG (4). Considerable overlapping ocurred between the UDPAG and UDPG peaks, eluted with 0.03 N sodium chloride in 0.01 N hydrochloric acid, owing probably to the relatively small amount of UDPAG present (*cf.* Cabib *et al.*) (4). The fractions corresponding to the UDPG peak were pooled and concentrated as previously described (4) and then chromatographed on blotting paper sheets (24) with ethanolammonium acetate of pH 3.8. The $R_{adenosine}$ values with this solvent were usually about 0.30 for GDPM, 0.45 for UDPG, and 0.60 for UDPAG. The blotting paper was prewashed with the same solvent and then with ethanol and distilled water successively. After chromatography, the ultraviolet-absorbing zone corresponding to GDPM was cut off, washed with ethanol to remove most of the ammonium acetate, and eluted with distilled water. The yield was about 25 μM.

Preparation 2—10 kilos of bakers yeast were submitted to the same initial steps as for the preparation of UDPG (2). The precipitate of the mercury salts of nucleotides (Step 2) was extracted with 1200 ml. of 1 M ammonium acetate. The insoluble fraction was blended with 1000 ml. of water and decomposed with hydrogen sulfide in the cold. After filtering, the resulting solution was aerated and neutralized. It was found by acid hydrolysis of small samples, followed by paper chromatography, that this fraction contained most of the acid-labile mannose. The extract (960 ml., containing about 8000 μM, calculated as above) was run on the Dowex 1 resin column as for Preparation 1 (see Fig. 1). The sustances corresponding to the different peaks were tentatively identified by their ultraviolet spectra, phosphate content, and chromatographic behavior, often by comparison with the nucleotides isolated previously (4). From Peak X about 340 μM of GDPM (calculated from the absorbancy at 260 mμ), contaminated with small amounts of UDPAG, were obtained. The substances were concentrated by adsorption and elution on charcoal and precipitated by stepwise addition of ethanol in the presence of calcium chloride (4). The calcium salt of GDPM, which is less

soluble, precipitates in the first case of UDPG and UDPAG (2, 4), are, however, substantially consistent with the proposed structure.

DISCUSSION

Buchanan *et al.* (16) have reported the presence of several nucleotide-bound hexoses in green plants and algae. The evidence indicated that glucose and galactose were present as UDP-glucose and UDP-galactose. A combined form of mannose, presumably GDPM, was also found in the same fraction.

The function of one of these nucleoside-pyrophosphate-sugar compounds as donor of the sugar moiety has been proved by Dutton and Storey (6). They found that UDP-glucuronic acid acts as a glucuronyl donor in the synthesis of glucuronides by liver enzymes. A somewhat similar rôle for UDPG in the synthesis of saccharose phosphate has been postulated by Buchanan *et al.* (16, 17). By analogy it may be assumed that GDPM and UDPAG function as donors of mannose and acetylglucosamine residues, respectively, and that they are involved in the synthesis of mannan and chitin, which are present in the yeast wall (18).

Methods

Methods were in general the same as those employed in previous papers (2, 4).

Paper chromatography of sugars was carried out with ethyl acetate pyridine-water (3, 19), or with phenol-ammonia (20). The chromatograms were sprayed with aniline phthalate (9) or benzidine-trichloroacetic acid (10).

For the chromatography of nucleotides and nucleosides, the ethanol-ammonium acetate mixtures already described (21) were used. In order to counteract the "tailing" shown by some of the compounds, a small amount of sodium Versenate (sodium salt of ethylenediaminetetraacetic acid) was added to the solvents (*cf.* Walker and Warren (22)). The concentration of chelating agent was 10^{-2} M for the solvent of pH 7.5 an 10^{-3} M for that of pH 3.8.

After examination for ultraviolet-absorbing spots with a Mineralight lamp, the chromatograms were often sprayed successively with bendizine-trichloroacetic acid and the Hanes and Isherwood molybdate reagent (12) to ascertain the position of sugar-and phosphate-containing substances.

Ionophoresis on paper of sugars and nucleotides was performed with an apparatus similar to that devised by Kunkel and Tiselius (23). A 0.05 M borax solution (pH about 9.2) was employed for sugars (11) and a 0.05 M ammonium acetate buffer of pH 3.8 for nucleotides. In chromatographic as well as in ionophoretic experiments, the position of the substance on fractions practically free from UDPAG. The preparation thus obtained was contaminated with a substance which migrated like GMP-5' on paper chromatography or ionophoresis. The latter procedure was selected for the preparative separation. Several fractions of the calcium salt of GDPM totaling 42 mg., were pooled and dissolved in water, after which the calcium was removed with ammonium oxalate. The supernatant liquid was submitted to ionophoresis on starch as described by Kunkel and Slater (25). A starch slab, 45 cm. long × 5 cm. wide × 0.5 cm. thick, moistened with 0.05 M ammonium acetate buffer of pH 3.8, was employed. The sample (0.7 ml.) was spotted at 4.5 cm. from the cathodic end along a transversal depression, which was afterwards replenished with barely moist starch. Ionophoresis was carried out during 6 hours under a potential of 750 volts. The position of the substances was ascertained with the Mineralight lamp. The GDPM band, which had migrated faster toward the anode, was cut off and extracted with two 75 ml. portions of water on a Büchner funnel. The water extracts were pooled, evaporated to dryness *in vacuo*, and left during 3 days at 35° in a vacuum desiccator containing sodium hydroxide and sulfuric acid to remove as much ammonium acetate as possible. The residue was redissolved in water, and GDPM precipitated as the calcium salt with aqueous ethanol. Yield, 21 mg.

Preparations 1 and 2 were chromatographically identical, but Preparation 2 was purer, as judged from the analytical data, and was used for all the determinations, except for the acid hydrolysis curve of phosphate and the identification of guanine.

Degradation Products of GDPM

Identification of mannose: for the quantitative tests, GDPM was hydrolyzed with 0.05 N

sulfuric acid during 15 minutes at 100°, the mixture was neutralized with 0.06 N barium hydroxide, and the nucleotides were precipitated by adding equal volumes of 5 per cent zinc sulfate and 0.3 N barium hydroxide. After centrifuging, mannose was determined in an aliquot of the supernatant liquid with an adaptation of the Schales and Schales ferricyanide method (26).

To obtain a solution of the sugar suitable for chromatography or ionophoresis, an aliquot of Sample B, previously used for the electrometric titration (see Table V), was passed successively through cation exchange and anion exchange resin columns (Dowex 50 and Amberlite IR-4B respectively). The solution was then evaporated *in vacuo*, and samples were taken for chromatography or ionophoresis on paper (Table I).

Guanosine Diphosphate — Aliquots of Sample B from the electrometric titration (Table V) were evaporated *in vacuo* and used for chromatography (Table II).

Guanosine Monophosphate. — An aliquot from Sample C, used for the electrometric titration (Table V), was centrifuged to separate the guanine which had precipitated, and the supernatant liquid was divided in two portions. One was evaporated *in vacuo* and used for paper chromatography (Table II), while the other was brought to pH 9 with 0.3 N barium hydroxide to precipitate inorganic phosphate. After centrifuging, the supernatant fluid was neutralized with sulfuric acid and centrifuged again. The resulting solution was evaporated *in vacuo* and used for the incubation with 5'-nucleotidase (see Table III).

The authors wish to express their gratitude to Professor A. R. Todd for a sample of synthetic guanosine-5'-phosphate, to Dr. L. A. Heppel for a preparation of 5'-nucleotidase, and to Dr. C. E. Cardini, Dr. H. Pontis Videla, Dr. J. L. Reissig, and Dr. R. E. Trucco for useful criticism. They are specially indebted to Dr. A. C. Paladini for help with the ionophoretic methods.

SUMMARY

The isolation of a mannose-containing nucleotide from yeast is reported. Mannose was identified by chromatography and ionophoresis on paper. The nucleotide was found to give an ultraviolet absorption spectrum nearly identical to that of guanosine and to contain two phosphate groups. Mannose could be liberated by mild acid hydrolysis. Further hydrolysis led to the removal of one phosphate group, leaving a substance which behaved like guanosine-5'-phosphate on paper chromatography and when treated with 5'-nucleotidase. After treatment with stronger acid, a substance with the properties of guanine was detected. The properties of the compound, including the titration curves, are consistent with those of a structure in which the terminal phosphate of guanosine-5'-pyrophosphate is joined to a mannosyl residue.

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JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 214, p. 149. 1955

THE BIOSYNTHESIS OF SUCROSE *

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Hassid, Duodoroff, and Putman (1-3) discovered an enzyme in certain bacteria (*Pseudomonas saccharophila*) which catalyzes the following reaction:

$$\text{Glucose-1-phosphate} + \text{fructose} \rightleftarrows \text{sucrose} + \text{inorganic phosphate}$$

The enzyme, which was named sucrose phosphorylase, bas not been found in plant tissues (1), and thus the mechanism of sucrose synthesis remains obscure. Evidence obtained from tracer experiments led Buchanan *et al.* (5, 6) to assume that in plants sucrose phosphate is formed from UDPG [1] and fructose-1-phosphate. This hypothesis stimulated work which led to the discovery of an enzyme which catalyzes sucrose synthesis according to the following reaction:

$$\text{UDPG} + \text{fructose} \rightleftarrows \text{sucrose} + \text{UDP} \qquad (1)$$

A brief note (7) reported the preparation of this enzyme from wheat germ and its presence in some other plant materials. Furthermore, the validity of Reaction 1 was proved by the equivalence between the disappearance of UDPG and the formation of UDP and sucrose. A more detailed study is reported in this paper.

Methods

Analytical — Sucrose was estimated by the resorcinol method of Roe (8). The volumes taken were reduced to one-fourth, and the color was measured at 490 mμ. In order to avoid the interference of fructose, the samples were heated 10 minutes at 100° after adding sufficient sodium hydroxide to make the concentration 0.2 N. While 2 μmoles of fructose were found to give no color with resorcinol after this treatment, sucrose remained unaffected even when the concentration of alkali was 0.5 N. High concentrations of monosaccharides yield some color, even after alkaline treatment, but this can be corrected by the use of suitable blanks.

The method of Kunitz and McDonald (9) was followed for protein estimation.

Substrates — UDPG and UDP were prepared as described previously (10). The sugars were commercial samples.

Estimation of Enzyme — The following components were mixed: 0.5 μmole of UDPG, 2 μmoles of fructose, 0.01 ml. of 2 M Tris buffer of pH 7.2, and variable amounts of enzyme; total volume, 0.15 ml. After 30 minutes at 37°, water was added to 0.5 ml., followed by 0.02 ml. of 5 N sodium hydroxide. After careful mixing, the tubes were heated 10 minutes at 100° and sucrose was estimated. An equal sample, in which UDPG was added after the incubation, and sucrose standards (0.1 to 0.2 μmole) were run at the same time.

Plant Material — Beet, sweet sorghum, or pea seeds were allowed to germinate 4 to 5 days at 30° under light on wetted cotton. The shoots were then ground, and the solids were removed by filtration through muslin. The liquid was then treated with ammonium sulfate, and the fraction obtained between 0.25 and 0.60 saturation was redissolved and dia-

* This investigation was supported in part by a research grant (No. G 3442) from the National Institutes of Health, United States Public Health Service, and by the Rockefeller Foundation.

[1] The following abbreviations are used: UDP for uridine diphosphate, UDPG for uridine diphosphate glucose, and Tris for tris (hydroxymethyl) aminomethane.

TABLE I

Purification of Enzyme

Fraction	Volume	Units[1]	Purity[2]
	ml		
I. Crude extract	70		~0.05
II. 1st ammonium sulfate ..	35	672	0.24
III. Manganous chloride	40	132	0.66
IV. 2and ammonium sulfate ..	20	108	1.16
V. Alumina, 1st supernatant	20	104	1.80
VI. „ 2nd „	20	72	2.40

1 unit is defined as the amount of enzyme catalyzing the formation of 1 μmole of sucrose in 30 minutes under the conditions described in the text.

2 Expressed in units per mg. of protein.

lyzed overnight in the refrigerator against distilled water. The protein content of the extracts was found to decrease with the age of the plantules.

Sugar cane shoots (2 to 3 cm. long) and roots were obtained from stem cuttings which had been kept in the laboratory on wetted cotton at 30°. The extracts were prepared as described above.

Purification of Wheat Germ Enzyme — 30 gm. of commercial wheat germ and 100 ml. of 0.05 M phosphate buffer of pH 7.2 were mixed in a blender. The suspension was centrifuged 15 minutes at 16,000 r.p.m., and the supernatant fluid was dialyzed with constant stirring at 5° during 4 to 5 hours. The liquid was centrifuged again as before (Fraction I, crude extract [Table I]).

The supernatant solution was made 0.5 saturated with solid ammonium sulfate, and the precipitate obtained by centrifugation was dissolved in half the volume of Fraction I of water and dialyzed overnight at 5° against distilled water (Fraction II). 0.1 volume of 1 M manganous chloride was added, and the suspension was stirred during 30 minutes at 0°. After centrifugation the supernatant fluid (Fraction III) was made 0.3 saturated with ammonium sulfate, and the precipitate was discarded. Ammonium sulfate to 0.5 saturation was added, and the precipitate was dissolved in half the volume of Fraction III of water and dialyzed during 1 to 2 hours in the cold with constant stirring (Fraction IV).

Alumina (Cγ) (usually 0.1 volume of a suspension containing 50 mg. of dry weight per ml.) was added to the liquid, and the precipitate was discarded. To the supernatant fluid more alumina was added (0.2 volume), and the precipitate was again discarded. The supernatant solution (Fraction VI) was the best preparation obtained (Table I).

Results

Properties of Enzyme — In crude extracts nearly all the activity could be recovered in the precipitate from adding acetic acid to pH 5. However, no appreciable purification was obtained by this procedure.

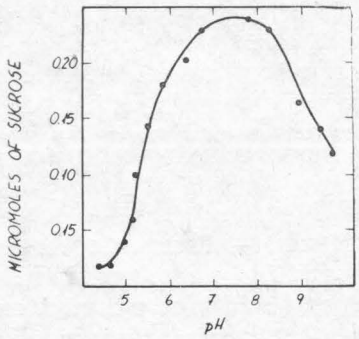

Fig. 1. pH optimum curve. System as described in the text with Tris or acetate buffer at 0.15 M final concentration. Incubated 15 minutes at 37°. The pH was determined on aliquots with a glass electrode.

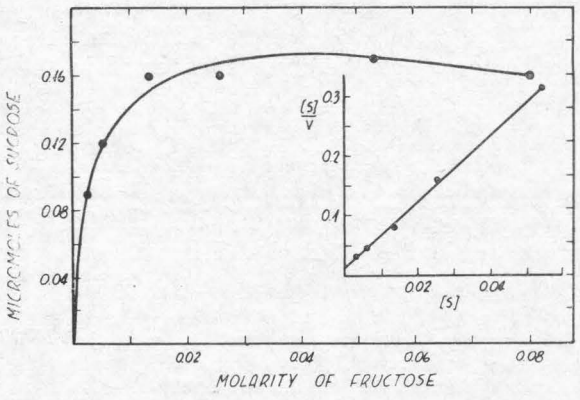

Fig. 2. Influence of fructose concentration. System as described in the text. The amount of purified enzyme corresponded to 0.18 mg. protein. Fructose concentration as indicated. Section at right, a Lineweaver-Burk (22) plot. $Km = 2.3 \times 10^{-3}$.

Heating 10 minutes to 60° led to nearly complete destruction of the wheat germ enzyme. Considerable destruction was found to take place on precipitation with acetone, even at low temperature. The enzyme could be stored for months in the frozen state without much loss of activity.

Inhibitors — The following substances did not affect the activity: arsenate, arsenite, fluoride, iodoacetate, citrate, or pyrophospate at 0.01 M concentration; calcium, barium, or magnesium ions at 0.05 M concentration; phlorizin, 8-hydroxyquinoline, or ethylenediaminetetraacetate at 0.2 saturation.

pH Optimum — As shown in Fig. 1, the highest activity was obtained at pH 7.2 with 0.15 M Tris buffer.

Substrate Concentration — The result of an experiment with different concentrations of fructose is shown in Fig. 2. If the amount of sucrose formed is taken as a measure of rate of reaction, calculation of the Michaelis constant was 2.3×10^{-3}. A 2-fold increase of UDPG concentration did not affect the rate of reaction.

Specificity — No substance reacting like sucrose was found to be formed when UDPG was replaced by glucose-1-phosphate, fructose-1-or 6-phosphate, UDP-acetylflucosamine (11), or guanosine diphosphate mannose (12), or when fructose was replaced by sorbose or by fructose-6-phosphate. However, the latter was true for only a few of the preparations obtained. This point will be dealt with in the following paper.

In other tests, the disappearance of UDPG was measured by a method based on its activity as cogalactowaldenase (10). When fructose was added to the wheat germ enzyme and UDPG, the disappearance of the latter was increased. No increased disappearance was observed if fructose was replaced by D-glucose, D-galactose, D-mannose, D- or L-arabinose, D-ribose, or inositol.

Reaction Product — The isolation of crystalline sucrose was not attempted, since it would have required considerable amounts of UDPG. However, the tests which have been carried out make it reasonably certain that the product is sucrose.

Paper chromatography of the reaction products revealed the presence of a substance which gave the R_F value of sucrose (Table II). This substance was absent in controls in which the fructose or the UDPG was added at the end of the incubation period. The substance behaved like sucrose when the papers were developed with the alkaline silver (13), resorcinol (14), or benzidine-trichloroacetic acid reagents (15).

In other experiments, the reaction product was isolated by paper chromatography. The substance was found to have no reducing power and to behave like sucrose during paper chromatography. After mild acid hydrolysis (5 minutes, pH 2, 100°) or after treatment with yeast invertase, glucose and fructose could be detected chromatographically.

With the solvents used for paper chromatography (ethyl acetate-pyridine-water (10:5:6) (16) and butanol-acetic acid-water (17) and with the reagents used for revealing the spots, sucrose can be easily distinguished from maltose, trehalose, lactose, and raffinose.

TABLE II

Paper Chromatography of Reaction Product

	R_{xylose}			
	Pyridine-ethyl acetate		Butanol-acetic acid	
Complete system ..	0.82	0.94	0.32	0.80
No UDPG *		0.93		0.82
„ fructose *		0.93		0.83
Sucrose		0.82		0.32
Glucose		0.87		0.58
Fructose		0.93		0.82
Maltose		0.72		0.20
Trehalose		0.75		

* Added at the end of the incubation period.

TABLE III

Acid Hydrolysis of Reaction Product

The samples (0.18 μmole) were heated at 100° in 1 ml. of 0.1 M glycine-hydrogen chloride buffer of pH 2.25. Samples of sucrose were run at the same time under identical conditions. The reducing power was measured with ferricyanide (23). Results in per cent hydrolysis.

	Time		
	2 min.	4 min.	6 min.
Sucrose	36	70	82
Reaction product ..	32	73	85

The rate of acid hydrolysis of a known sample of sucrose was compared under identical conditions with a sample of the reaction product; both substances hydrolyzed at the same rate (Table III).

Reversibility — In order to detect the reversibility, the back-reaction was investigated by starting with 5 μmoles of sucrose, 2 μmoles of UDP, and enzyme. The mixture was deproteinized with trichloroacetic acid, and, after extracting the latter with ether, the samples were chromatographed on paper with an ethanol-ammonium acetate solvent of pH 7.5 (18) containing 0.01 M ethylenediaminetetraacetate. A spot having the same mobility as UDPG was visible under ultraviolet light. After extraction of the substance from the paper, UDPG was estimated by its cogalactowaldenase activity and absorption at 260 mμ. The amount obtained was about 0.05 μmole. Control samples in which one of the reactants was omitted during the incubation gave no UDPG spot or cogalactowaldenase activity.

Many attempts to obtain a precise figure for the equilibrium constant were carried out by starting with known mixtures of reactants and products. The reducing power or the sucrose content of the samples was measured before and after enzyme action. A small correction had to be applied, owing to the liberation of reducing power from sucrose, which occurred even with the most purified enzyme preparations. Many experiments were carried out in this manner, but the results were not consistent and the value for $K =$ (sucrose $\times$ UDP) / (UDPG $\times$ fructose) varied from 2 to 8 at 37° and pH 7.4 in different experiments.

Distribution — The detection of the enzyme in some plant tissues is difficult, owing to the presence of sucrase. However, it has been possible to obtain extracts from many sources which catalyze the formation of sucrose, and in general seeds were the best materials for the preparation of the enzyme. Quantitative measurements were carried out with some extracts, and the results were as follows (in micromoles of sucrose formed in 30 minutes per mg. of protein): beet shoots 0.6 to 1.0, sweet sorghum shoots 0.7 to 4.0, sugar cane shoots 0.25, sugar cane roots 2.4 to 3.0, pea shoots 0.35. Qualitative tests for the enzyme were positive with the following materials: pea, pine, and fenugreek seeds, corn germ, potato sprouts, and barley shoots. Negative or non-reproducible results which may be attributed to interfering enzymes were obtained with sugar beet and cane sugar leaves and with beet roots.

DISCUSSION

The equilibrium constant of the sucrose phosphorylase reaction has been found to be 0.053 at pH 6.6 and 30° (4). This displacement in favor of the monosaccharides makes the enzyme appropriate for the utilization of sucrose, and this is probably its main function in *P. saccharophila*. In contrast, the equilibrium of the reaction starting with UDPG and fructose is in favor of sucrose synthesis. Accurate values have not been obtained, but the $\Delta F°$ at 37° can be estimated to be about -1000 as compared to $+$ 1770 for sucrose phosphorylase.

The enzyme has been found in many plant materials, and hence we may conclude that it catalyzes a reaction which is fairly general in the plant kingdom. Thus, the enzyme is probably involved in the formation of sucrose by pea extracts detected by Turner (19). However, the synthesis of sucrose from fructose and UDPG is not the only pathway in plants since, as reported in the following paper, another enzyme catalyzes the synthesis of sucrose phosphate from fructose-6-phosphate and UDPG, and the reaction product can be transformed into sucrose by phosphatase action.

According to modern nomenclature (20), the enzyme might be named UDPG-fructose transglycosylase. Following the suggestion of von Euler (21) that enzymes might receive the name of the substrates which they synthesize, with the ending changed to *ese,* another possible name is saccharese. This shorter alternative is used currently in this laboratory.

SUMMARY

A wheat germ enzyme which catalyzes the reaction

$$\text{UDPG} + \text{fructose} \rightleftarrows \text{sucrose} + \text{UDP}$$

has been studied.

Methods for its estimation and purification are described, as well as the conditions for its maximal activity. The equilibrium of the reaction was found to be displaced in favor of sucrose synthesis, the value of K being about 5 at 37° and pH 7.4. The enzyme was detected in several plant tissues.

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JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 214, p. 157, 1955

THE BIOSYNTHESIS OF SUCROSE PHOSPHATE *

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The enzymic formation of sucrose from UDPG [1] and fructose has been described in the preceding paper (1). While some preparations of the enzyme were found to be almost devoid of action of fructose-6-phosphate, other extracts catalyzed the formation of free sucrose either from fructose or from its phosphate. Since these extracts contain phosphatase, it seemed likely that the action on fructose phosphate was the sum of Reaction 1 plus Reaction 2.

$$\text{Fructose-6-phosphate} + H_2O \rightarrow \text{fructose} + \text{inorganic phosphate} \quad (1)$$

$$\text{UDPG} + \text{fructose} \rightleftarrows \text{sucrose} + \text{UDP} \quad (2)$$

However, further work has shown that it is possible to obtain enzyme preparations which catalyze Reaction 3.

$$\text{UDPG} + \text{fructose-6-phosphate} \rightleftarrows \text{sucrose phosphate} + \text{UDP} \quad (3)$$

Phosphatase acts on sucrose phosphate according to Reaction 4.

$$\text{Sucrose phosphate} \rightarrow \text{sucrose} + \text{phosphate} \quad (4)$$

Crude extracts of wheat germ catalyze all four reactions, and it has not been possible to obtain a complete separation of the enzymes. Nevertheless, with some of the preparations the rate of Reaction 3 was 2 or 3 times faster than that of Reaction 2, and sucrose phosphate has been obtained in amounts which have allowed the determination of its structure with reasonable certainty.

* This investigation was supported in part by a research grant (No. G-3442) from the National Institutes of Health, United States Health Service, and by the Rockefeller Foundation.

1. The following abbreviations are used: UDP for uridine diphosphate, UDPG for uridine diphosphate glucose. Tris for tris (hydroxymethyl) aminomethane, and TPN for triphosphopyridine nucleotide.

Methods

Preparation of Enzyme — The method of separation of the enzymes acting on fructose and on fructose phosphate was based on the observation that the fructose phosphate enzyme has a greater tendency to become insoluble when dialyzed against water. The procedure described in the preceding paper was used, but with some changes. The mixing with a blender was omitted, thus making centrifugation at high speed unnecessary, as well as the first dialysis. The wheat germ, 100 gm., was suspended in 300 ml. of 0.05 M phosphate buffer of pH 7.1 and left standing at 5° during 1 hour. The paste was centrifuged 15 minutes at 3000 r.p.m. Without dialyzing, the supernatant fluid was treated with ammonium sulfate, manganous chloride, and ammonium sulfate again, as previously described. The precipitate obtained in the second ammonium sulfate treatment (Fraction IV) was dissolved in the smallest possible amount of water and dialyzed overnight at 5° against several changes of distilled water. The precipitate was separated by centrifugation and washed three to four times by suspending in 1 ml. of distilled water and centrifuging. The precipitate was then extracted successively with 2 ml. of 0.05 M and 0.1 M ammonium sulfate. The pooled extracts were dialyzed overnight. The precipitate was washed and extracted successively with 2 ml. of ammonium sulfate at the concentrations shown in Table I (Preparations 1 to 4). In some cases, the precipitation by dialysis and extraction had to be repeated in order to obtain a preparation with a low content in phosphatase and in UDPG fructose transglycosylase.

TABLE I

Purification of Enzyme

The activity is expressed in micromoles of total sucrose (free plus esterified) formed in 30 minutes per mg. of protein under the conditions described in the text.

Preparation	Protein	Activity on		$\frac{A}{B}$
		Fructose (*A*)	Fructose 6-phosphate (*B*)	
	mg. per ml.			
Fraction IV	23	1.16	0.20	5.8
1. 0.05 M $(NH_4)_2SO_4$ extract	40	0.70	0.65 [1]	1.06
2. 0.1 " " "	22	0.50	0.90 [2]	0.55
3. 0.1 " " "	10	0.40	1.20	0.33
4. 0.1 " " "	4.4	0.90	1.10	0.82

[1] and [2] 65 and 82 per cent, respectively, of the total sucrose formed was esterified.

Analytical — Sucrose phosphate was estimated as described by Cardini *et al.* (1) with a sucrose standard. In order to distinguish sucrose from its phosphate, the samples were analyzed with and without precipitation with the zinc sulfate-barium hydroxide reagent (2). The Fiske and Subbarow (3) method was used for the estimation of phosphate.

Fructose-6-phosphate was estimated by using a preparation of glucose-phosphate dehydrogenase (4), which also contained phosphohexose isomerase.

Substrates — Fructose-6-phosphate was a commercial sample. A synthetic sucrose phosphate was prepared by direct phosphorylation of sucrose as described by Neuberg and Pollak (5).

Fructose-1-phosphate was synthesized according to Raymond and Levene (6).

Estimation of Enzyme — The procedure was as described in the previous paper, but the pH of the buffer was 6.4 and fructose-6-phosphate was used instead of fructose.

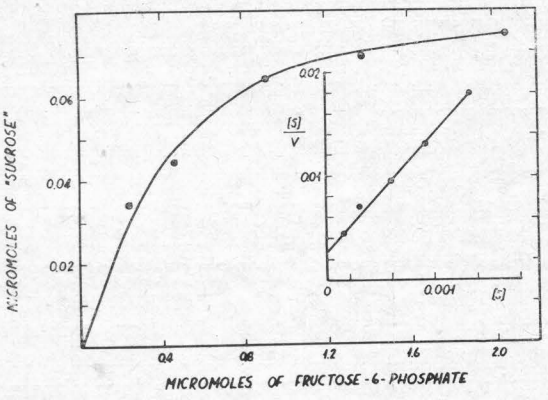

FIG. 1. — Influence of fructose-6-phosphate concentration. System as described in the text. The amount of purified enzyme corresponds to 0.05 mg. of protein. Fructose-6-phosphate concentration as indicated. Incubated 30 minutes at 37°. Section at right, a Lineweaver-Burk (18) plot. $Km = 2.2 \times 10^{-3}$.

Results

Properties of Enzyme — A comparison of "sucrose" formation from free fructose and fructose-6-phosphate with different preparations of the enzyme has shown that the ratio of activities can vary from 5.8 to 0.33 (Table I), and this is considered proof that two different enzymes are involved. Even the best preparations still contained some phosphatase, and hence the reaction product was usually a mixture of sucrose phosphate and free sucrose. This contamination decreased considerably the yield of sucrose phosphate, thus rendering the isolation more difficult. The substitution of fructose-1-phosphate for fructose-6-phosphate as a subtrate for the enzyme led to a great decrease in the yield of "sucrose", most of which way free. Nevertheless, there was a small amount which appeared to be esterified, but this point could not be clarified because the sample of fructose-1-phosphate contained a small amount of the 6 ester and because of the presence of phosphatase. This point will have to be investigated further.

The effect of changing the concentration

of fructose-6-phosphate is seen in Fig. 1. Calculation of the apparent Michaelis constant gave a value of 2.2×10^{-3}. Fig. 2 shows the influence of changing the pH. The optimum was at pH 6.4.

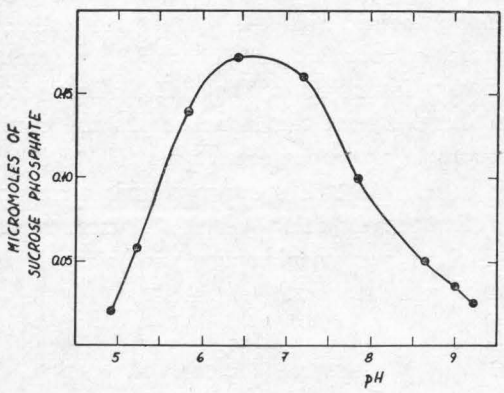

FIG. 2. — pH optimum curve. System as described in the text with Tris or acetate buffer at 0.15 M final concentration. Incubated 60 minutes at 37°. The pH was determined on aliquots with a glass electrode.

Isolation of Reaction Product — After many preliminary trials with paper chromatography and electrophoresis, the separation of the reaction product was carried out by anion exchange chromatography. The method of Khym and Cohn (7) with slight modifications was used. The effluents were analyzed by the resorcinol method (8), with and without previous alkaline treatment, and by ultraviolet absorption at 260 mμ. The procedure was as follows: The reaction mixture was prepared by mixing 160 μmoles of UDPG, 500 μmoles of fructose-6-phosphate, and 8 ml. of enzyme (total volume, 40 ml.) and incubating 1 hour at 37°. The proteins were coagulated by heating and filtered. The solution was passed through Dowex 50 and neutralized with ammonia. It contained 40 μmoles of "sucrose phosphate" in 155 ml. This was percolated into a column of Dowex 1 in the chloride form (4.15 sq. cm. × 12 cm.), followed by 300 ml. of 0.001 M ammonia. All the organic phosphate was retained in the column. Gradient elution was carried out with an apparatus similar to that described by Alm, Williams, and Tiselius (9) by adding 0.03 M ammonium chloride to a 500 ml. mixing chamber filled with a solution containing 0.025 M ammonium chloride and 0.01 M sodium borate. Free sucrose and some ultraviolet-absorbing substances emerged first, and, after 450 ml. had passed through the column, "sucrose phosphate" began to emerge and was collected in 315 ml. Fructose-6-phosphate was retained even after several liters of this solvent had been passed through the column. The tubes containing "sucrose phosphate" were pooled, and the solution was passed through Dowex 50, neutralized with ammonia, and evaporated to dryness under vacuum. The borate was then removed by 3-fold addition of methanol and vacuum distillation. The product was dissolved in water, passed through Dowex 50, and neutralized with calcium carbonate. After filtering, the liquid was evaporated to dryness. Extraction with 95 per cent ethanol removed the calcium chloride, leaving the calcium salt of "sucrose phosphate". The latter was dissolved in 1 ml. of water, clarified by centrifugation, and precipitated with 6 volumes of ethanol, washed with ethanol, and dried. About 17 mg. of a white powder were obtained. The "organic phosphate" content was 1.4 μmoles per mg. (theoretical for the anhydrous Ca salt = 2.44). The fructose-phosphate ratio was 0.90.

Properties of Reaction Product. — The product obtained was found to have no reducing power or ultraviolet absorption at 260 mμ. The estimation of fructose by Roe's (8) resorcinol method corresponded to 90 per cent of the organic phosphate. It is known that fructose esters give less color than free fructose; thus Lutwak and Sacks (10) obtained values as low as 58 per cent for fructose-6-phosphate and 87 per cent for fructose diphosphate, and there is always some uncertainty in respect to the purity of the samples.

The sustance is stable to alkali. Thus, no phosphate was liberated by heating in 0.5 N alkali during 30 minutes, in contrast to fructose phosphates (Table II). The fructose content estimated by the resorcinol method did not change after heating 10 minutes in 0.2 N alkali during 10 minutes. The rate of removal of the phosphate group by 1 N acid at 100° is similar to that of fructose-6-phosphate and clearly different from that of fructose-1-phosphate and of synthetic sucrose phosphate, which is a mixture of isomers (Table II).

The appearance of reducing power during acid hydrolysis was measured (Fig. 3). Under those conditions, sucrose was hydrolyzed about 5 times faster than the "reaction product". The "reaction product" does not reduce TPN when incubated with glucose dehydrogenase plus isomerase (Table III). However, reduction of TPN takes place with the product of hydrolysis.

The results of experiments by paper chromatography (Table IV) may be summarized as follows: Mild acid hydrolysis gives glucose spot and a phosphoric ester spot, but no free fructose. Treatment with kidney phosphatase or with a crude extract of wheat germ in the presence of Mg^{++} results in a substance migrating like sucrose. Hydrolysis of this "sucrose" with acid or with yeast invertase gives glucose and fructose. Other experiments showed that the "reaction product" can be separated from fructose-6-phosphate by paper chromatography with several solvents. Thus, with methanol-formic acid (11) or with ethanol ammonium acetate of pH 3.8 (12), it migrates at about 75 per cent the rate of fructose-6-phosphate. About the same separation could be obtained by paper electrophoresis with borate buffer (13). These procedures were not used for the isolation of the substance because there was overlapping with ultraviolet-absorbing substances.

TABLE II

Acid and Alkaline Hydrolysis

The results represent the per cent of organic phosphate liberated.
Alkaline hydrolysis was carried out with sodium hydroxide in silver test-tubes.

	1 N sulfuric acid				0.5 N alkali	
	Heating at 100°					
	10 min.	20 min.	30 min.	60 min.	15 min.	30 min.
Fructose-1-phosphate	77	87	100		92	100
Fructose-6-phosphate			13	22	76	78
Reaction product			10	25	0	0
Synthetic sucrose phosphate			42	52	0	0

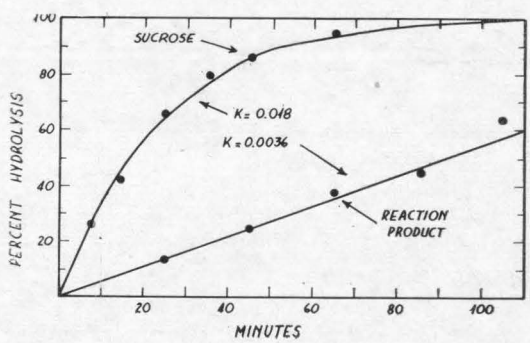

FIG. 3. — Acid hydrolysis of reaction product. The curves represent the theoretical values calculated for a first order reaction ($\log_{10}$ and minutes). The points correspond to the results obtained by incubating 0.15 μmole of substance at 37° in 0.9 N hydrochloric acid. After the specified time, the samples were neutralized and the reducing power was measured by the ferricyanide method (19). Samples which were heated 4 minutes at 100° in 0.9 N acid were considered to give the value for 100 per cent hydrolysis.

TABLE III

Reduction of TPN by Reaction Product after Hydrolysis

The substances were mixed with with 0.15 μmole of TPN, 0.4 ml. of 1 per cent sodium bicarbonate, 3 mg. of *Zwischenferment*, and water to 2.5 ml. The TPN reduced was calculated from the increase in absorbancy at 340 mμ. Results in micromoles.

Substance	TPN reduced (*A*)	Substance added (1) (*B*)	$\frac{A}{B}$
Fructose-6-phosphate	0.08	0.07	1.14
Fructose-1-phosphate	0.004	0.13	0.03
Reaction product	0.006	0.14	0.04
Reaction product hydrolyzed 2	0.068	0.074	0.92

1 Calculated from the organic phosphate content.
2 Heated 5 minutes at 100° in 0.1 N acid.

TABLE IV

Paper Chromatography of Reaction Product

Solvent, *n*-butanol-pyridine-water, 6:4:1.5. Benzidine-trichloroacetic acid reagent (20) as developer.

	*R*xylose		
	Experiment I	Experiment II	Experiment III
Reaction product		0.04	
" " + acid hydrolysis*		0.1, 0.75	
" " + kidney phosphatase	0.56		0.59
" " + crude wheat germ	0.55		
" " + kidney phosphatase invertase			0.76, 0.84
Sucrose	0.56	0.52	0.59
" + invertase			0.76, 0.85
Fructose	0.82	0.83	0.84
Glucose		0.75	0.75
Fructose-6-phosphate		0.04	

* Heated 5 minutes in 0.1 N acid at 100°.

The reaction product was found to remain unaffected after treatment with yeast or honey invertase. The latter finding was unexpected, since honey invertase (14) is believed to be specific for the glucose moiety of the substrate.

DISCUSSION

It can be concluded that the reaction product is sucrose with a phosphate group at position 6 of the fructose moiety, because dephosphorylation with phosphatase produces a substance behaving like sucrose, and mild acid hydrolysis gives free glucose and a fructose ester which behaves like fructose-6-phosphate when treated with acid or with isomerase plus glucose dehydrogenase. However, it should be pointed out that the samples obtained were only about 60 per cent pure, as judged by the phosphate content of the calcium salt. Further purification was not attempted because only small amounts were available. A point of interest is that sucrose phosphate is more stable to acid than is free sucrose. Thus, the ratio of the hydrolysis constants for the glycosidic links, (K for sucrose) / (K for sucrose phosphate), is 5 in 0.9 N acid at 100°. It was found previously (15) that the ratio of the hydrolysis constants of the phosphate in position 1, (K for glucose-1-phosphate) / (K for glucose-1, 6-diphosphate), is 4 in 0.25 N acid at 37°. In both cases, the phosphate group at position 6 stabilizes the substituents at the other end of the molecule.

The sucrose phosphate detected by buchanan (16) was believed to yield fructose-1-phosphate by hydrolysis, and hence it would be different from the product described in this paper.

A study of the distribution of the two enzymes which lead to the synthesis of sucrose would be of interest for plant physiology, but is difficult to carry out, owing to the presence of interfering enzymes (phosphatase and sucrase). Studies on green leaves with labeled substrates (17) have shown that the labels are introduced into both halves of sucrose before appearing in the free monosaccharides. This would prove that free fructose is not involved directly in the synthesis, were it not for the fact that the same experiments demonstrated that most of the sugars in leaves are stored in a metabolically inert compartment. Thus the introduction of the label in free fructose at the site of synthesis might have been faster than it appeared to be in these experiments.

SUMMARY

A wheat germ enzyme which catalyzes the following reaction has been studied:

UDPG + fructose-6-phosphate → UDP + sucrose phosphate

It is concluded that the reaction product is sucrose with a phosphate group at position 6 of the fructose moiety, because dephosphorylation with phosphate yields a substance behaving like sucrose and mild acid hydrolysis gives free glucose and a fructose ester which behaves like fructose-6-phosphate when treated with acid or with isomerase, glucose dehydrogenase, and TPN.

The glycosidic linkage of sucrose phosphate was found to be more stable to acid than was that of sucrose.

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BIOCHIMICA ET BIOPHYSICA ACTA
Vol. 20, p. 33, 1956

ENZYMES ACTING ON GLUCOSAMINE PHOSPHATES *

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Uridine disphosphate acetylglucosamine was first isolated from yeast [1] and has been detected in mammalian liver [2 3 4 5] and hen oviduct [6] A specific pyrophosphorylase has been found [5] to transform it into uridine triphosphate and acetylglucosamine-1-phosphate, so that the latter is likely to be a normal metabolite in mammals. In order to make this ester available for enzymic studies, a chemical method for its synthesis has been developed, following a procedure inspired by that used by Cori, Corowick and Cori [7] for the preparation of glucose-1-phosphate.

Acetylglucosamine-1-phosphate was first tested with an enzyme from *Neurospora* and found [8] to be converted to acetylglucosamine-6-phosphate. This enzyme which is activated by the 1,6-disphosphates of glucose or acetylglucosamine has been studied by Reissig [9]. It has now been observed that similar changes are catalyzed by mammalian enzymes thus giving rise to acetylglucosamine-6-phosphate. This substance which has been previousty obtained by the enzymic acetylation of glucosamine 6-phosphate [8 10] has been found to be converted into fructose-6-phosphate by kidney enzymes. But the most unexpected finding was that it catalyses the enzymes transformation of glucosamine-6-phosphate into fructose-6-phosphate and ammonia. Furthermore the latter reaction was found to be reversible so that it affords a possible route to hexosamine synthesis different from that found in *Neurospora* [8] where glutamine is involved. Previous work on hexosamine metabolism has been reviewed by Dorfman [11] and Kent and Whitehouse [12].

* This investigation was supported in part by a rescarch grant (No. G 3442) from the National Institutes of Health, U. S. Public Health Service.

METHODS

Analytical

The following methods were used. Phosphate. Fiske and Subrarow [13], protein, Kunitz and McDonald [14]; fructose, Roe [15]: glucosamine, Blix [16]; *ammonia*, Conway [17]; for acetlyglucosamine a modification of the Morgan and Elson method [18] was used with a standard of acetylglucosamine prepared as described by Roseman and Ludowieg [19]. With purified enzymes deproteinization was unnecessary. For acetylglucosamine-1-phosphate which does not give the test directly-proteins were precipitated with 5 % trichloracetic acid. After centrifugation, 0.5 ml. of supernatant was heated 10 minutes at 100°C in orden to hydrolyze the phosphate group. After cooling, 0.15 ml of 1.*M potassium* borate was added and the procedure continued as described by Reissig *et al* [18] *A* larger amount of borate (0.15 instead of 0.1 ml) was used in order to neutralize the acid.

When it was necesary to distinguish free hexosamines from their phosphoric esters (Table III) the latter were precipitated by adding zinc sulfate and barium hydroxide solutions as described by Somogyi [20].

Estimation of the enzymes

The test system for measuring the disappearance of acetyglucosamine-6-phosphate contained 0·1 μmole of substance 0.02 ml of 1*M* tris-hydroxymethylaminomethame buffer of pH 7.7 and the enzyme.

For glucosamine -6-phosphate the system contained 0·1 μmole of *N* glucosamine-6-phosphate, 0.02 ml of 1 *M* tris buffer of pH 8 4, 0.02 μmole of acetylglucosamine-6-phosphate and the enzyme.

For both tests the total volume was 0.05 ml and the incubation time: 15 minutes at 37°C *A* unit was defined as the amount of enzyme causing the disappearance of 25 % of the substrate in 15 minutes.

Preparation of acetyglucosamine-I-phosphate

This substance was prepared by making trisilver phosphate react with chlorotetraacetylglucasamine. An amorphous bromo derivative was used in many cases until Dr. R. Jeanloz suggested the use of the chloro compound which can be obtained crystaline.

a-I-chloropentacetyglucosamine. The procedure described by Baker *et al.*[21] was used with minor modifications. Pentacetylglucosamine (6.2 g) prepared with zinc chloride as catalyst as described by Levene [22] was added to a solution obtained by mixing 104 ml of dry ethyl ether saturated with HCl at 0°C. 18.7 ml

of acetic acid anhydride and 6 ml of glacial acetic acid. After keeping for 2-3 days at 0ºC with occasional shaking, the solid was dissolved. The solution was concentrated *in vacuo* below 15ºC. The solid was then dissolved in chloroform and treated as described by BAKER *et al.*[21].

Phosphorylation. 2.9 g of chloropentaacetylglucosamine were mixed with 1.2 g of trisilver phosphate[23] and 60 ml of dry benzene. This mixture was heated in a water bath and about 5 ml of the benzene was distilled in order to remove traces of water. After this the heating was continued during 15 minutes, under reflux with shaking. The solid changed from yellow to white and the liquid became brownish.

The mixture was filtered hot through filter aid (celite) and washed with hot benzene.

Hydrolysis. The benzene solution was concentrated to dryness *in vacuo* and the solid was dissolved in 30 ml of methanol containing 1.2 ml of 5 *N* sulfuric acid. After keeping it for 30 minutes at 37º C the pH was adjusted to about 8.9 (thymol blue) with 10 *N* sodium hydroxide. The pH was kept alkaline during a few hours by occasional addition of sodium hydroxide. An excess of 50 % barium acetate was added followed by 30 ml of ethyl ether. After leaving it overnight at 5ºC the mixture was centrifuged. The precipitate was extracted several times with water. The pooled water extracts contained 730 µmoles of labile phosphate.

Purification. A solution containing about 100 µmoles of labile phosphate adjusted to pH 8 was poured onto a column (90 cm × 1.8 cm^2) of Dowex 1 of 10 % crosslinkage in the chloride form. Gradient elution was carried out as described by ALM *et al.*[24]. A solution of 0.005 *M* HCl in 0.1 *M* $CaCl_2$ was allowed to enter into a 250 ml mixing chamber filled wtih water. The fractions (5 ml) were analysed for acetylglucosamine, after heating for 10 minutes at 100ºC in 0.1 *N* acid, and for labile phosphate. Several well separated peaks appeared. The first was free acetylglucosamine. The second had a ratio acetylglucosamine/labile phosphate of 1.7 to 1.9 and consisted mainly of a diester. The third peak which was the largest contained acetylglucosamine-1-phosphate. Finally a small peak containing unidentified substances appeared.

The fractions corresponding to the third peak were pooled, neutralized with solid $Ca(OH)_2$, filtered, concentrated *in vacuo* to 1-2 ml and precipitated with 3 volumes of ethanol. If necessary the precipitation was completed by the addition of ethyl ether. The solid was washed several times with ethanol in order to remove the calcium chloride, washed with ethyl ether and dried. Usually the solid was dissolved in a small amount of water, centrifuged and precipitated with ethanol. The yield was about 50 % of the labile phosphate introduced into the column. The ratio acetylglucosamine/phosphate was about 1 and the purity with respect to dry weight was 84 %.

The rotatory power was measured on 0.3 ml of solution containing 39 µmoles of he calcium salt and the concentration was checked by phosphate estimation. $[\alpha]_D = +107$.

Preparation of glucosamine-6-phosphate

Glucosamine was phosphorylated with ATP by a procedure similar to that described by BROWN[25]. The following mixture was incubated at pH 8: 220 µmoles of glucosamine hydrochloride, 120 µmoles of ATP, sodium salt, 1 ml of 0.1 *M* magnesium sulfate and 1 ml of Lebedew juice (prepared by extracting dry yeast with 3 volumes of 0.1 *M* sodium bicarbonate during 24 hours at 5º C) and water to complete 10 ml. After 1 hour at 35º C the proteins were coagulated by heating and centrifuged off .

The filtrate was then poured into a column 1.8 cm^2 X 100 cm of Dowex-1 (X 10) in the acetate form. Elution was carried out by a gradient obtained by allowing 0.05 *N* acetic acid to flow into a mixing chamber containing 250 ml of water. The fractions were analysed by the method of BLIX[16].

The first fractions contained glucosamine while the phosphate ester appeared later and was well separated from the former. The last fractions of glucosamine phosphate were slightly contaminated with a substance absorbing at 200 mµ and were rejected.

The pooled fractions of glucosamine phosphate were concentrated *in vacuo* to about 3 ml, the pH was adjusted to 7.2 with barium hydroxide and 3 volumes of ethanol were added. The precipitate was separated, washed with ethanol and ether and dried. The yield was 60-70 % of the glucosamine used, and the product was 80 % pure.

Preparation of N-acetylglucosamine-6-phosphate

Glucosamine-6-phosphate was treated with acetic anhydride as described by ROSEMAN[26]. The whole reaction mixture including the resin used for the acetylation was poured on top of a column of Dowex-1 chloride. Displacement from the column was effected gradient-wise as described for acetylglucosamine-1-phosphate, but using 0.15 *N* HCl. The fractions which gave a ratio total phosphate/acetylglucosamine of one were pooled and the calcium salt was obtained as described for the 1-phosphate. The yield was rather low owing to losses occurring in the precipitation with ethanol.

In some experiments acetylation was carried out at 0ºC with a slight excess of acetic anhydride in aqueous-pyridine solution. The results were essentially the same as with the other procedure.

N-propionylglucosamine-6-phosphate

The procedure was the same as for the acetyl derivative but propionic acid anhydride was used.

Purification of the enzyme acting on the 6-phosphates

Pig kidneys obtained frozen from the slaughterhouse yielded active extracts even after several weeks storage in the frozen state. The cortex was homogenized with a blendor in 3 volumes of water and the mixture was centrifuged at 3000 r.p.m. in the cold for 10 minutes (crude extract).

To 80 ml of the crude extract 40 ml of ammonium sulfate solution (50 g percent w/v) were added. After 10 to 15 minutes at 5ºC the precipitate was centrifuged off and discarded. To 100 ml of the supernatant 20 ml of ammonium sulfate solution were added. The precipitate was separated, dissolved in water and dialysed 4-5 hours at 5ºC (Fraction A). Ammonium sulfate solution (0.2 vol) was added to the supernatant of the previous step. The precipitate was redissolved and dialysed (Fraction B).

Fraction B was treated with a solution of yeast nucleic acid adjusted to pH 7 (100 mg per g of protein). By cautious addition of dilute HCl followed by contribution, several fractions were obtained (B_1 B_2, *etc.*). The precipitates were suspended in water and neutralized. The results are shown in Table I.

TABLE I

Purification of the enzymes

Fraction	*Volume ml*	*Protein mg/ml*	*Glucosamine-6-P*		*Acetylglucosamine-6-P*		*Ratio A / B*
			Units/mg A	*Total units*	*Units/mg B*	*Total units*	
Crude extract	80	53	10	43000	0.75	3200	13.3
Fraction A	5	60	30	9000			
Fraction B	3.5	50	40	7000	3.8	650	10.5
Fraction B_1	0.23	50	30	350			
Fraction B_2	0.35	70	75	1800			
Fraction B_3	0.30	58	196	3500	5.0	88	39
Fraction B_4	0.30	46	93	1300			

RESULTS

Prosperties of acetylglucosamine-I-phosphate

The molecular rotation of I-phospho sugars is known to be comparable to that of the methylglycosides (*cf.* LELOIR [27]). For methyl-N-acetylglucosaminide the values given by Neuberger and Pitt Rivers [28] are + 24,675 for the α- and 10.105 for the β-anomer. The acetylglucosamine-1-phosphate prepared as described here gave a value of + 36,000 so that it is the α-anomer. The product has been used by Dr. REISSIG [9] in a study of the phosphoacetylglucosaminemutase of *Neurospora* and several preparations were found to be over 90 % converred into the 6 phosphate. The substances does not reduce sugar reagents and gives the Morgan and Elson reaction only after acid hydrolysis.

TABLE II

Acid hydrolysis of acetylglucosamine-1-phosphate

Time (hours)	*Acetylglucosamine-1-phosphate*		*Glucose-1-phosphate*	
	Percent hydrolysis	*10³K*	*Percent hydrolysis*	*10³K*
1	26.6	2.2	37.8	3.3
2	41.0	1.9	60.5	3.4
3	58.2	1.7	76.2	3.4
4	58.2	1.6	79.0	2.8
5	67.8	1.7	87.0	2.9
6	75.4	1.7	92.3	3.1
18	100.0	—	100.0	—
	Mean	1.8		3.1

Inorganic phosphate was estimated after incubating the samples at 37°C in 1 *N* sulfuric acid. The formula used was: $K = (t_2 - t_1)^{-1} \log_{10} (100 - x_1) / (100 - x_2)$. The time was taken in minutes. No detectable amounts of glucosamine were formed.

The results of acid hydrolysis in 1 *N* acid at 37°C are shown in Table II. It may be observed that the acetylglucosamine ester is slightly more stable than that of glucose. However it is much more labile than glucosamine-1-phosphate [29] and galactosamine -1-phosphate [30]

The action of enzymes on acetylglucosamine-1-phosphate

On incubation of the 1-phospho ester with crude kidney or liver extracts it was observed that a part was converted to free acetylglucosamine while another part was transformed into substances which did not give the Morgan and Elson test even after acid hydrolysis. The formation of acetylglucosamine-6-phosphate as an intermediate in this reaction could be detected only by purification of the crude extracts or as shown in Table III by the use of an inhibitor. Such a selective inhibition of acetylglucosamine-6-phosphate-disappearance could be obtained with acetate without affecting the activity on the 1-phosphate. Under these conditions about half of the decrease in acetylglucosamine-1-phosphate could be accounted for by the increase in the 6-phosphate. These results are shown in Table III. Other test were carried out with an enzyme prepared by a method based on the first steps of the purification of phosphoglucomutase as described by NAJJAR [31]. The results in Table IV show that the process is activited by magnesium ions and slightly activated by glucose disphosphate. In that experiment the results of the estimation were checked with a specific enzyme method for acetylglucosamine-6-phosphate. In the other experiments rat muscle or liver extracts were found to catalyse the conversion of the 1-to 6-phosphate at similar rates.

The action of enzymes en the 6-phosphates

Crude preparations of pig kidney catalyse the disappearance of acetylglucosamine-6-phosphate. Glucosamine-6-phosphate also disappears provided small amounts of acetylglucosamine-6-phosphate are also added. As shown in Table I both activities may be detected after some purification but the ratio of the two varies from 13 to 39. The action on the non-acetylated substance was always higher than on the acetylated.

TABLE III

The action of kidney extracts on acetylglucosamine-1-phosphate

	Δ *in micromoles*	
	No acetate	*With acetate*
Acetylglucosamine-1-phosphate (c—a)	— 0.053	— 0.060
Free acetylglucosamine (b)	+ 0.012	+ 0.017
Acetylglucosamine-6-phosphate (a—b)	+ 0.002	+ 0.033

Incubation of 0.05 ml of crude kidney extracts (1 vol of water used) 0.01 ml of 0.36 *M* glycerophosphate buffer pH 7.4, 0.01 ml of 1 *M* sodium acetate (pH 7.4) and 0.08 μmole of acetylglucosamine-1-phosphate Estimations were as follows: (a) direct estimation of acetylhexosamine, (b) same in the supernatant obtained after precipitation with zinc sulfate and barium hydroxide, (c) as (a) but after removing protein with 5 % trichloracetic acid and heating 10 minutes at 100°C.

TABLE IV

The conversion of acetylglucosamine-1-phosphate to the 6-phosphate

	μ *moles of acetylglucosamine-6-phosphate*	
	Chemical method	*Enzymic method*
Complete system	0.083	0.08
No glucose diphosphate	0.076	—
No magnesium	0.045	—

The complete system contained 0.01 ml of 0.36 *M* glycerophosphate buffer pH 7.4, 0.005 ml of saturated solution of 8'-hydroxyquinoline, 0.01 ml. of 0.1 *M* magnesium chloride, 0.005 μmole of glucose diphosphate, 0.17 μmole of acetylglucosamine-1-phosphate and enzyme. Incubated 1 hour at 37°C, total volume 0.08 ml. The enzyme was prepared from pig kidney by adjusting the crude extract to pH 5, precipitating the supernatant with 0.65 saturated ammonium sulfate and dialysing. Estimations by chemical method as in Table III. For the enzymic method the activating power on glucosamine-6-phosphate disappearance was compared with a standard of acetylglucosamine-6-phosphate using a purified enzyme.

The pH optima are shown in Fig. 1.

Free glucosamine or acetylglucosamine were not acted upon by the enzyme preparations.

Many experiments we carried out in order to detect a cofactor. The enzyme was submitted to prolonged dialysis against ethylenediaminetetracetate solutions, to precipation with 0.1 *N* acid from ammonium sulfate solutions and to treatment with anion exchange resins but no organic or inorganic ion requiriment could be detected.

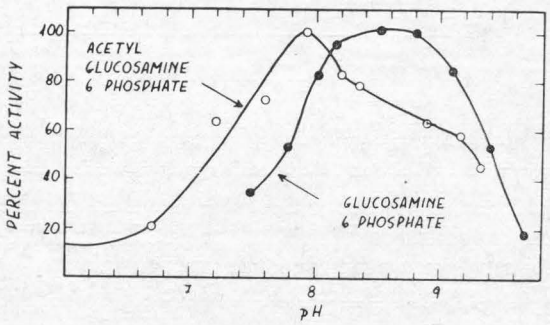


FIG. 1. — pH optima. Condtions as described for test system with Tris HCl and Tris-maleate, buffers prepared as described by GOMORI 35. The pH was checked in aliquots with a glass electrode.

The action of acetate

As shown in Table V acetate nearly suppresses acetylglucosamine-6-phosphate disappearance while it does not affect appreciably that of glucosamine-6-phosphate. The results with propianate or butyrate were similar to those obtained with acetate while formate showed no action.

TABLE V

The action of acetate

Substrate	*Addition*	*Substrate* (γ *mole*)
Acetylglucosamine-6-phosphate	none	0.09
Acetylglucosamine-6-phosphate	acetate	0.02
Glucosamine-6-phosphate	none	0.14
Glucosamine-6-phosphate	acetate	0.14

Conditions as described for test system with 0.2 *M* acetate.

The action of acetylglucomasine-6-phosphate on glucomasine phosphate disappearance

As shown in Fig. 2 glucosamine-6-phosphate is not transformed in the absence of acetyl-

glucomasine-6-phosphate. The effect of the latter is catalytic since 0.009 μmole can produce the disappearance of 0.05 μmole of glucosamine-6-phosphate. Many substances have been tested as possible substitutes for acetylglucosamine-6-phosphate. The results were negative with: acetylglucosamine, acetamide, UDP-acetylglucosamine, acetate, acetate plus-ammonia, hexose-6-phosphates, acetylglycine, acetyltriptophane, acetylcholine, pyruvate, citrate and α-Ketoglutarate. With acetylglucosamine-1-phosphate some activation was obtained using crude extracts but none with purified preparations.

Only one substance was found to have the activity of N-acetylglucosamine-6-phosphate and that was N-propionylglucosamine-6- phosphate. This ester was also found to disappear on incubation with the ensyme. The quantitative results were very similar with the acetyl and propionyl derivatives.

te the reaction was faster so that the interference of traces of isomerase was smaller. Fig. 3 shows the result of such an experiment. Glucosamine-6-phosphate disappeared rapidly with a concomitant rise in the fructose values and in the sum fructose-6 plus glucose-6-phosphate as deteminated whit glucose-6-phosphate dehydrogenase plus isomerase and TPN. After some minutes the fructose values decreased slowly, as was expected, owing to the presence of isomerase in the enzyme preparation.

In another experiment with a preparation that was nearly free from isomerase the results were as follows:

	Δ
Glucosamine-6-phosphate	− 0.046
"fructose"	+ 0.045
Ammonia	+ 0.048

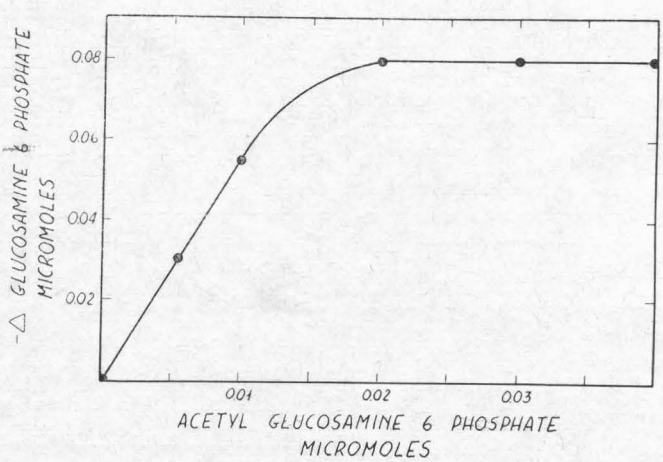


FIG. 2. — The action of acetylglucosamine-6-phosphate on glucosamine-6-phosphate disappearance. Conditions as described for test system, but with variable amounts of acetyl glucosamine-6-phosphate.

The reaction products

Analysis by paper chromatography of the products obtained from acetylglucosamine-6-phosphate with a crude enzyme preparation, and after treatment with phosphatase, revealed the presence of glucose, fructose and in some cases of a substance reacting like heptulose [32]. Hexose phosphate isomerase was dificult to remove from the preparations but in one case a quantitative accumulation of fructose ester from acetylglucosamine-6-phosphate was obtained. With glucosamine-6-phosphate as substrate

It seems clear therefore that the primary reaction products are fructose-6-phosphate and ammonia.

Reversible formation of glucosamine-6-phosphate

The incubation of fructose-6-phosphate and ammonia with the enzyme and small amounts of acetylglucosamine-6-phosphate led to a definite increase in "glucosamine" as estimated by the BLIX method. No increase ocurred

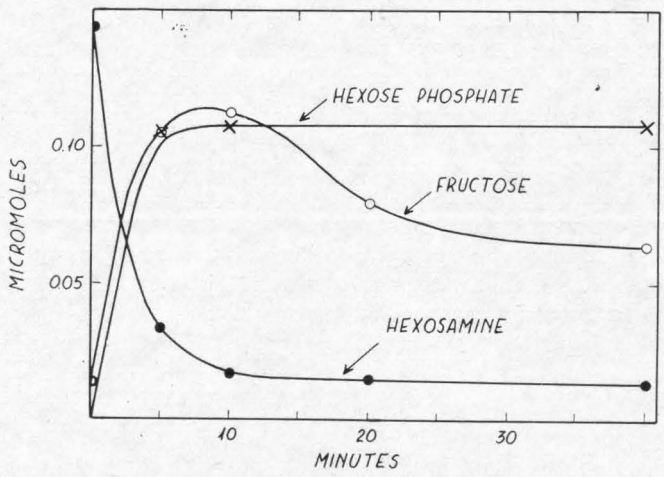


FIG. 3. — Chemical changes during the transformation of gldcosamine-6-phosphate. The reaction mixture contanied 0.15 μmole of glucosamine-6 phosphate, 0.04 μmole of acetylglucosamine-6-phosphate, 0.04 ml of 1 *M* tris buffer of pH 8.4 and 0.04 ml of enzyme precipitated twice with nucleic acid. Total volume 0.1 ml. Temperature: 37°C.

if the ammonia, fructose phosphate or acetylglucosamine-6-phosphate were omitted or if ammonia was substituted by glutamine or asparagine. The results were also negative if free fructose, glucose, xylose or ribose were added instead of fructose-6-phosphate. A representative experiment is shown in Table VI. These results were taken as an indication that the reaction can be reversed and measurements of the equilibrium constant were carried out, starting with known reaction mixtures and estimating the changes in glucosamine phosphate produced by the enzyme. One suchs experiment is shown in Table VII. The values obtained for *K* in moles/liter varied from 0.12 to o.18. In other experiments the results were more variable (0.04 to 0.19). Owing to analytical errors and to some uncertainly regarding the true molarity of the substrates perhaps the results should only be taken as indicating the order of magnitude of *K*. Thus the fructose-phosphate concentration was calculated assuming that isomerase converted it into the equilibrum mixture which contains 66 % of glucose-6-phosphate. However, it was clear in all the experiments that similar values for *K* are obtained in the forward and in the reverse reaction.

TABLE VI

Synthesis of glucosamine-6-phosphate

	"Glucosamine" formed μ moles
Complete system	0.014
No ammonium sulfate	0.002
No acetylglucosamine-6-phosphate	0.002
Glutamine instead of ammonium sulfate	0.002
Asparagine instead of ammonium sulfate	0.002

The complete system contained: 0.5 μmole of fructose-6-phosphate 1.5 μmoles of ammonium sulfate, 0.02 μmole of acetylglucosamine-6-phosphate, 0 01 ml of 2 *M* tris buffer and 0.27 mg of enzyme preparation (B_1 Table I). Total volume 0.05 ml, 30 minutes at 37°C. The glucosamine values obtained on samples at t_0 were substrated.

TABLE VII

Equilibrium constant

Substances added (μ moles)			Δ	*K*
Fructose-6-P	*Ammonia*	*Glucosamine-6-P*	*Glucosamine*	
0.326	1.0	0	+ 0.0128	0.16
0.326	1.0	0.0085	+ 0.0058	0.15
0.326	1.0	0.0170	+ 0.0016	0.12
0.163	2.0	0	+ 0.011	0.18
0.163	2.0	0.0085	+ 0.0057	0.15
0.163	2.0	0.0170	− 0.002	0.16

Incubation of fructose-6-phosphate with ammonium sulfate and glucosamine-6-phosphate; 0.02 ml-tris buffer 1 *M* pH 8.4; 0.02 mg enzyme (B_3, Table I) final volume 0.05 ml., 10 min at 37°C, $K = [NH_2]$ - [Tructose-6-phosphate] / [glucosamine-6-phosphate] in moles/liter. The concentration of fructose-6-phosphate was taken as 1/3 of theorical amount in order to correct for the presence of isomerase.

TABLE VIII

The action of extracts from different organs on glucosamine-6-phosphate

Organ	*Activity units/mg protein*
Kidney	10.0
Brain	2.2
Intestine	1.0
Liver	0.5
Lung	0.4
Heart	0
Brewars yeast	0

The rat organs were homogenized in 2 vol. of water at 0°C and centrifuged. Test with glucosamine-6-phosphate were carried out as described for the test system.

Distribution of the glucosamine-6-phosphate enzyme (s)

As shown in Table VIII, kidney is the richest source of enzyme, followed by brain, intestine, liver and lung. No activity was detected in heart or in yeast. The same extracts were tested for the rate of the reverse reaction starting with fructose phosphate and ammonia and essentially similar results were obtained.

DISCUSSION

The first step in the transformation of acetylglucosamine-I-phosphate catalysed by animal tissues has been found to be:

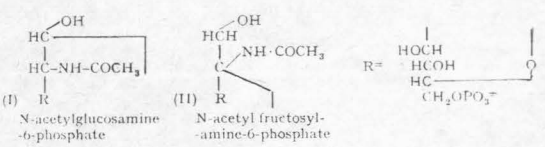
acetylglucosamine-1-phosphate $\rightleftharpoons$ acetylglucosamine-6-phosphate

It remains to be decided whether the change is brought about by phosphoglucomutase or by a specific enzyme. Evidence showing that two enzymes are present in *Neurospora* extracts has been obtained by REISSIG [9] who separated some fractions which were more active on the acetylglucosamine ester than on glucose phosphate, and other fractions which behaved inversely. He also detected phosphoacetylglucosaminemutase activity in a highly purified rabbit muscle phosphoglucomutase.

As to the transformations of the 6-esters the reported facts can be rationalized by the following reactions:

Other formulations involving a cofactor can be written but no supporting evidence was found despite many efforts. Reactions (I) + (3) would be responsible for the disappearance of acetylglucosamine-6-phosphate. Acetate would inhibit reaction (3) -Glucosamine -6-phosphate would be transformed through reactions (I) + (2) and thus the necessity of catalytic amounts of acetylglucosamine-6-phosphate would be explained.

As to the nature of the substance X it is attractive to suppose that it is N-acetylfructosylamine-phosphate (II). In this case reaction (1) would be similar to an Amadori rearrangement, *i.e.* the conversion of an aldose (I) to a ketose (II) derivative. After transacetylation (Reaction (2)) the product would be fructosylamine phosphate, which is presumably an unstable substance that descomposes into fructose phosphate and ammonia. It may be mentioned that fructosylamine does not appear to be stable since it has never been prepared and when fructose reacts with ammonia it is the isomer, glucosamine, which has been obtained (HEYNS AND MEINECKE [33], CARSON [34]).



ACKNOWLEDGEMENT

Our thanks are due to Dr. E. CABIB for a preparation of glucose dehydrogenase, to Dr. E. RACKER for suggesting nucleic acid precipitation as a means of removing isomerase and to MARGRETE OXHOLM for technical assistance.

SUMMARY

The chemical synthesis of α-acetylglucosamine-1-phosphate and some of its properties are described. From kidney or liver, enzymes have been obtained which

$$\text{acetylglucosamine-6-1'} \rightleftharpoons X \quad (1)$$

$$\text{glucosamine-6-P} + X \rightleftharpoons \text{acetylglucosamine-6-P} + \text{fructose-6 P} + NH_3 \quad (2)$$

$$X \rightarrow \text{fructose-6-P} + NH_3 + \text{acetate} \quad (3)$$

catalyse the following reactions:

$$\alpha\text{-acetylglucosamine-1-phosphate} \rightleftharpoons \text{acetylglucosamine-6-phosphate} \quad (1)$$

$$\text{N-acetylglucosamine-6-phosphate} \rightleftharpoons \text{fructose-6phosphate} + NH_3 + \text{acetate} \quad (2)$$

$$\text{glucosamine-6-phosphate} \rightleftharpoons \text{fructose-6-phosphate} + NH_3 \quad (3)$$

Reaction (1) was found to be activated by magnesium ions. The enzyme(s) responsible for reactions (2) and (3) were purified and it was observed that (3) requires catalytic amounts of N-acetylglucosamine-6-phosphate (or of the N-propionyl derivative) and that it is reversible. The possible mechanism of the reactions is discussed.

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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Vol. 79, p. 6340, 1957

BIOSYNTHESIS OF GLYCOGEN FROM URIDINE DIPHOSPHATE GLUCOSE [1]

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Previous work has shown that UDPG [2] acts as glucose donor in the synthesis of trehalose phosphate [3], sucrose [4], sucrose phosphate [5] and cellulose [6].

TABLE I

Analytical Changes

The complete system contained: 0.5μ mole of UDPG, 0.33 μmole of glycogen, tris-(hydroxymethyl) -aminomethane buffer of *p*H 7.4, 0.01 *M* ethylenediaminetetraacetate and 0.02 ml. of enzyme. Total volume 0.07 ml. Incubation: 45 min. at 37°. The enzyme was prepared from an aqueous extract of rat liver by acidification to *p*H 5. The precipitate was washed four times with acetate buffer of *p*H 5 and redissolved in buffer. Results in μmoles.

	Δ UDP (a)	Δ glycogen
Complete system	0.22	0.27
No UDPG	0	− 0.03

a Estimated with pyruvate kinase 7. *b* Measured with a phenol-sulfuric acid reagent after precipitation with ethanol 8 and expressed as glucose.

When UDPG is incubated with a liver enzyme and a small amount of glycogen the chemical changes shown in Table I were found to take place. Approximately equal amounts of UDP and of glycogen were formed. Such an increase in glycogen could only be detected with liver preparations freed from amylase. Other preparations obtained by ammonium sulfate precipitation contained amylase and therefore lost their glycogen. With such enzymes no UDP formation took place unless a primer was added. As shown in Table II glycogen and soluble starch acted as primers whereas glucose and maltose were ineffective. Several mono-, di- and oligosaccharides and hexose phosphates were tested with negative results. Treatment of glycogen with α-amylase destroyed its priming capacity. It can be concluded that UDPG acts directly as a glucose donor to glycogen and that the reaction is thus similar to polysaccharide formation from glucose 1-phosphate with animal phosphorylase which requires a primer of high molecular weight. The enzyme was found in the soluble fraction of liver and became very unstable after purification.

TABLE II

Primer Requirement

System as in Table I, but glycogen omitted. The enzyme (0.01 ml.) was obtained by precipitation with 1.6 *M* ammonium sulfate followed by dialysis. Incubated 60 min. at 37°.

Additions	Δ UDP (μmoles)
None	0
0.1 mg. glucose	0
0.2 mg. maltose	0
0.4 mg. glycogen	0.08
0.4 mg. soluble starch	0.06

1 This investigation was supported in part by a research grant (No. G-3442) from the National Institutes of Health, U. S. Public Health Service, and from Laboratorios de Investigación de E. R. Squibb & Sons Argentina, S. A.

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 74, p. 84, 1958

PHOSPHORYLATION OF ACETYLHEXOSAMINES [1]

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INTRODUCTION

In a previous paper (1), a liver enzyme system which leads to the formation of free acetylgalactosamine from uridine diphosphate-acetylglucosamine, was described. In experiments designed to discover the further fate of acetylgalactosamine, it was found that liver and kidney enzymes catalyze its phosphorylation by adenosine triphosphate (ATP). Furthermore, a similar process was found to take place with acetylglucosamine as substrate. Phosphorylation of acetylglucosamine with *Escherichia coli* extracts has been reported by Soodak (2) and by Faulkner and Quastel (3). The phosphorylation of nonacetylated hexosamines has been studied by several workers. Thus glucosamine has been found to be phosphorylated by ATP and hexokinase to glucosamine 6- phosphate (4, 5). Similarly, galactosamine is transformed into galactosamine 1-phosphate by an enzyme presumably identical with galactokinase (4).

METHODS

Analytical

The following methods were used: Blix (7) for hexosamines, Morgan and Elson as modified by Reissig *et al.* (8) for acetylhexosamines, Kunitz and McDonald (9) for proteins, Fiske and SubbaRow (10) for phosphate, and a slight modification of the method of Schales and Schales (11) for reducing power.

Substrates

Galactosamine and acetylgalactosamine were purified as described previously (1). Acetylglucosamise 6-phosphate and acetylgalactosamine 1-phosphate were obtained by acetylation of glucosamine 6-phosphate (17) and galactosamine 1-phosphate (1), respectively.

1 This investigation was supported in part by research grants: (No. G-3442) from the National Institutes of Health, U. S. Public Health Service, and from Laboratorios de Investigación de E. R. Squibb & Sons, Argentina.

Paper Chromatography

The procedure described by Cardini and Leloir (1) was used for free acetylhexosamines, and that of Paladini and Leloir (12) for the esters.

Preparation of the Enzymes

The organs were homogenized in 3 vol. of 0.15 *M* KCl containing 0.001 *M* ethylenediamine tetraacetate. The enzymes were prepared from rat liver or kidneys following the procedure described by Roy (13) for the preparation of the sulfate activating system. It consists of a high-speed centrifugation in which the small particles which decompose ATP are removed, followed by ammonium sulfate precipitation between the following molar concentrations: *A*: 0-1.7, *B*: 1.7-2.3, *C*: 2.3-2.9. The precipitates were dissolved in water to a concentration of 80-100 mg. protein/ml.

Estimation of the Enzymes

The disappearance of acetylhexosamine was measured after precipitating the proteins and phosphate esters with $ZnSO_4$ and $Ba(OH)_2$. Usually the reaction mixture contained (in micromoles) 0.1 of acetylglucosamine (or acetylgalactosamine), 0.2 of sodium ATP, 0.3 of $MgCl_2$ and 0.03 ml. of 0.25 *M* tris (hydroxymethyl) aminomethane buffer of pH 7.1 (or 8.2 for acetylgalactosamine) and 0.01 ml. of enzyme solution. Total volume 0.10 ml. Incubation was usually carried out for 2 hr. at 37°, after which 0.1 ml. of 100 % $ZnSO_4$ and 0.1 ml. of 0.3 *M* $Ba(OH)_2$ were added (14). After centrifuging 0.2 ml. of the supernatant was taken for acetylhexosamine analysis. The difference in acetylhexosamine content between a complete sample and one incubated without ATP was taken as the amount esterified. When the esterification of acetylgalactosamine was measured, the precipitation of the phosphate esters with the zinc and barium solutions could be omitted without modifying the results.

Purification of the Reaction Products

Preliminary experiments showed that the reaction products could not be purified using ion-exchange resins, because they were not adsorbed due to the relatively large amounts of inorganic salts. It was observed, that the reaction product could be adsorbed on charcoal from acid solution, and eluted on raising the pH. This observation provided the basis for the method of purification described below. The reaction mixture was made up as follows: 50 μmoles of

acetylhexosamine, 100 μmoles of ATP, 60 μmoles of magnesium chloride, and 5 ml. of enzyme solution. Total volume 20 ml. The pH was adjusted to 7.1 with sodium hydroxide when acetylglucosamine was the substrate and to 8.2 for acetylgalactosamine. The enzyme fraction used corresponded to *A* or *B* or *C*, respectively. After incubating for 2 hr. at 37°, 3 vol. of ethanol was added, the precipitate was filtered off, and the filtrate was freed from ethanol by evaporation under reduced pressure to about one-tenth of the original volume. The solution was then adjusted to pH 4 and poured into a column of 1.8 cm. diameter and 17 cm. long, containing a mixture of 8 g. charcoal (Norit A) and 8 g. filter aid (Hyflo Super-Cel). Before the experiment the column was washed with 0.05 *N* formic acid until the effluent was acid to bromocresol green.

Once the sample has been adsorbed on the charcoal, elution was carried out gradientwise as described by Alm, Williams, and Tiselius (15). A 250-ml. mixing chamber contained three parts of 0.05 *N* formic acid and one part of 0.05 *N* ammonium hydroxide. The inflowing solution was 0.05 *N* ammonium hydroxide. Samples of 6-10 ml. were collected. The sulfate and inorganic phosphate appeared in the first tubes, while the acetylhexosamine esters emerged after 200-300 ml. of solution had passed through the column and when the pH was about 7. In the experiments where acetylglucosamine was the substrate, the samples were analyzed directly for acetylhexosamine. When the substrate was acetylgalactosamine, acid was added to the samples to 0.5 *M*. They were then heated for 5 min at 100°, neutralized with the calculated amount of NaOH solution, and then analyzed for acetylhexosamine. The samples containing acetylhexosamine were pooled, concentrated under reduced pressure, and then stored for several days in a vacuum desiccator over sodium hydroxide and sulfuric acid in order to remove the ammonium formate. The yield was about 60 %. In the experiments with the ester from acetylgalactosamine which is alkali-stable, the evaporation was carried out at pH 7-8, while with the acetylglucosamine ester the pH was maintained around 6.

Results

Conditions for Maximal Activity

As shown in Fig. 1, the greatest activity was obtained with 0.003 *M* Mg++. The pH optima were 7.1 and 8.2, respectively, for acetylglucosamine and acetylgalactosamine (Fig. 2).

Properties of the Enzyme

As shown in Table I, the activity of the different fractions obtained by precipation with ammonium sulfate is not parallel for the two substrates. The activity on acetylglucosamine is greater in fraction A, and that on acetylgalactosamine in fraction C. No activity on galactosamine was detectable when tested under the same conditions with the rat kidney enzyme. The fraction most active on acetylglucosamine (A) was the least active on glucosamine, so that it seems that the enzymes acting on the acetylated hexosamines are different from those acting on the nonacetylated substances. Under the conditions of the tests, acetylglucosamine 6-phosphate did not disappear when incubated with any of the enzyme fractions. However, after dialysis B catalyzed the disappearance of acetylglcosamine 6-phosphate as has been described previously (17). These reactions are inhibited by ammonium ions, and that is why they cannot be detected with undialyzed preparations. As

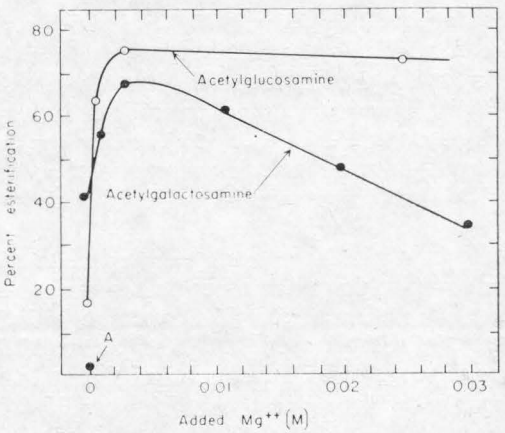

FIG. 1. — Effect of Mg++ concentration. Assays as described in text. Enzyme fraction A was used for acetylglucosamine, and fraction B for acetylgalactosamine. The amounts of ATP were 0.4 and 1.6 μmoles, respectively. Incubation time was 1 hr. Point A corresponds to a reaction mixture containing 0.01 *M* ehtylenediamine tetraacetate.

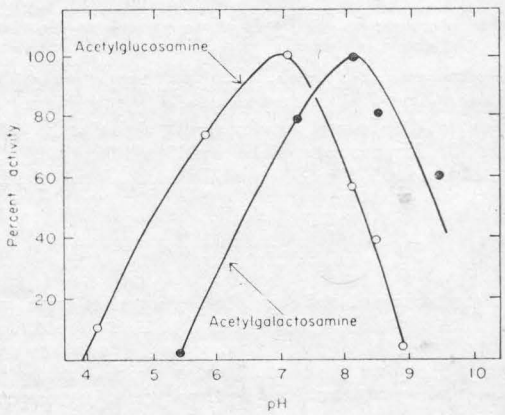

FIG. 2. — pH optimum: The pH was measured on aliquots with a glass electrode. The buffers were tris (hydroxymethyl) aminomethane with maleate (pH 5-7) or chloride (pH 7-9) (16).

TABLE I

Activity on Different Substrates

Methods as described in text. The rate of esterification was calculated from the linear part of a time curve.

Kidney fractions	Micromoles esterified/hr. Acetylglucosamine	Acetylgalactosamine	Glucosamine
A	0.06	0	0
B	0.05	0.03	0.007
C	0.024	0.06	0.014

to the stability of the enzymes, they could be stored for a month or more at −10°C. without great loss in activity. Dialysis overnight led to complete inactivation of the acetylgalactosamine enzyme. No reactivation could be obtained by adding heated extracts. The enzyme acting on acetylglucosamine could be dialyzed overnight at 0° without apreciable loss of activity.

Activity in Different Organs

Table II shows the results obtained with extracts of different organs. Kidney extracts were most active on acetylgalactosamine, followed by heart, spleen, and liver. The activity on acetylglucosamine was slightly lower than with acetylgalactosamine as substrate. The negative finding of Faulkner and Quastel (3) with brain extracts and acetylglucosamine as substrate may have been due to the high ATP-descomposing activity of their extracts.

TABLE II

Activity in Different Organs

Assays as in Table I except that the amount of ATP was 1.0 μmoles and the final volume was 0.2 ml. The enzyme solutions were obtained as described in the text, except that only one precipitation was carried out with 2.9 *M* ammonium sulfate.

	Rate of esterification in micromoles/hr./mg. protein Acetylgalactosamine	Acetylglucosamine
Kidney	0.10	0.06
Heart	0.07	0.05
Spleen	0.06	0.02
Liver	0.05	0.04
Brain	0.03	0.03
Lung	0.01	0.01

Properties of the Reaction Products

Acetylglucosamine. When the tests for enzyme activity were carried out without precipitation of the phosphate esters, no change was detectable in the amount of acetylhexosamine. This shows that the reaction products gives this reaction similar to free acetylglucosamine.

Analysis of the product obtained after purification with charcoal gave the following results: (total phosphate taken as 1.0): inorganic phosphate, 0; Morgan and Elson reaction (with acetylglucosamine as standard) 1.1; reducing power (with acetylglucosamine as standard) 0.9.

After hydrolysis of the phosphate group with phosphate, acetylglucosamine was identified by paper chromatography on borate-treated paper.

When run on paper with the ethanol-ammonium acetate solvent of pH 3.8, the product gave the same *Rf* as a sample of acetylglucosamine 6-phosphate obtained by acetylation of glucosamine 6-phosphate with acetic anhydride. Moreover, both substances gave approximately the same results when tested as activators of the deamination of glucosamine 6-phosphate with kidney enzymes (17). Another test which was carried out consisted in adding the deaminase together with glucose 6-phosphate dehydrogenase, isomerase, and triphosphopyridine nucleotide. Thus the formation of fructose 6-phosphate could be followed by reduction of triphosphopyridine nucleotide in a spectrophotometer. In this test the two above-mentioned preparations were indistinguishable. The phosphorus liberated in 0.2 *N* alkali at 100° in 3 min. was 60 %. A sample of glucose 6-phosphate which was run at the same time gave 64 %. All the properties of the reaction product which have been investigated are those of acetylglucosamine 6-phosphate. This substance has been prepared previously by Brown (18) by enzyme acetylation of glucosamine 6-phosphate, and by Roseman and Ludowieg (19) and by Leloir and Cardini (17) using acetic anhydride. Maley and Lardy (20) have published a purely chemical method of synthesis.

Acetylgalactosamine. In this case the result of activity tests was the same with or without precipitation of the phosphate esters, thus showing that some change occurred in the group involved in the reaction with *p*-dimethylaminobenzaldehyde. Analysis of the product obtained by purification with charcoal gave the following results (total phosphate taken as 1.0):

	Direct	After acid hydrolysis
Inorganic phosphate	0	1.00
Morgan and Elson reaction (acetylgalactosamine as standard)	0	1.1
Reducing power (acetylgalactosamine as standard)	0	1.1

The substance was compared with a sample of acetylgalactosamine 1-phosphate obtained by acetylation of galactosamine 1-phosphate with acetic anhydride (1). When run on paper with ethanol-ammonium acetate solvent of pH 3.8, the two substances were indistinguishable. After acid hydrolysis, acetylgalactosamine was detected by paper chromatography. The rate of acid hydrolysis in 1 *M* acid at 37° was measured and, as shown in Fig. 3, the substance is hydrolyzed about three times more rapidly than α glucose 1-phosphate. The lack of reducing power before hydrolysis shows that the phosphate is in position one. Moreover, the molar rotatory power ($\alpha M = \alpha_D \times$ mol. wt) measured in water was 52,000, which is the same within the experimental errors as the value of + 54,000 found (1) for a preparation obtained by acetylation of α galactosamine 1-phosphate. Thus all the properties of the substance obtained by enzymic phosphorylation of acetylagalactosamine indicate that it is α-acetylgalactosamine 1-phosphate.

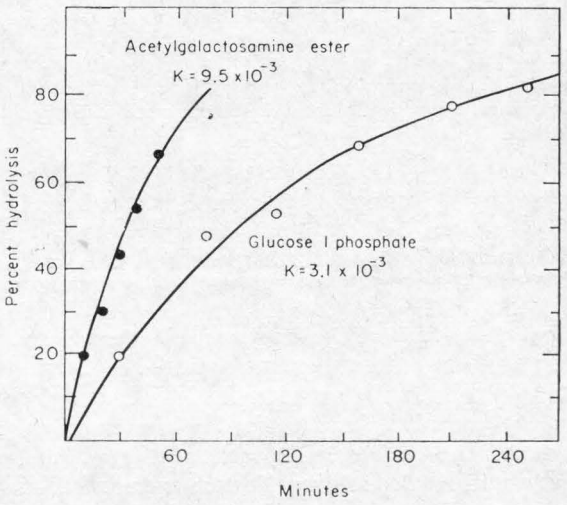

FIG. 3. — Acid hydrolysis of the ester obtained from acetylgalactosamine. After incubation in 1 *N* sulfuric acid at 37° the samples were analyzed for inorganic phosphate. The value of the hydrolysis constant *K* was calculated using $\log_{10}$ and minutes.

SUMMARY

The phosphorylation of acetylglucosamine and acetylgalactosamine by adenosine triphosphate catalyzed by extracts of rat organs has been studied. The reaction products had the properties of acetylglucosamine 6-phosphate and acetylgalactosamine 1-phosphate, respectively. The enzymes appear to be different from other kinases.

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 81, p. 508, 1959

BIOSYNTHESIS OF GLYCOGEN FROM URIDINE DIPHOSPHATE GLUCOSE[1]

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INTRODUCTION

The biosynthesis of glycogen from UDPG[5] with a liver enzyme has been reported previously (1). The reaction has been investigated further using a partially purified preparation form rat muscle with which the general properties of the system have been studied.

METHODS

Analytical

Glycogen was estimated by the phenol-sulfuric acid method (2), after digestion with KOH and ethanol precipitation (3). A sample of glycogen prepared as described by Somogyi (4) was used as standard. Its concentration was checked against glucose using the anthrone method (5). UDP was estimated as described by Cabib and Leloir (6), but halving the amounts of reagents. UDPG was measured spectrophotometrically with a partially purified UDPG dehydrogenase (7). Phosphorylase was estimated as described by Cori *et al.* (8). Protein was measured by the methods of Kunitz and McDonald (9) and of Warburg and Christian (10). Amylase activity was determined under the same conditions as the glycogen-forming enzyme but without UDPG or G-6-P. After deproteinization with Ba $(OH)_2$ and $ZnSO_4$, the reducing substances were measured according to Park and Johnson (11). G-6-P was estimated spectrophotometrically (12). Radioactivity was measured with a gas-flow counter. Radioactive sugars in paper chromatograms were located with a silver reagent (13) and then eluted, plated, and counted. Approximately half of the added counts were detected after this treatment.

1 This investigation was supported in part by a research grant (Nº G-3442) from the National Institutes of Health. U.S. Public Health Service.

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4 Investigador of the Comisión Nacional de Energía Atómica. mica.

5 Abreviations used: UDPG: uridine diphosphate glucose; UDP; uridine diphosphate; G-1-P: glucose-1-phosphate; G-6-P: glucose-6-phosphate; Tris tris (hydroxy methly) aminomethane; EDTA: ethylenediamine tetraacetate.

Substrates

UDPG was isolated from yeast ast described by Pontis *et al.* (14). UDPG labeled in the glucose moiety was prepared by incubating C^{14}G-6-P with UDPG and a crude *Saccharomyces fragilis extract* (15) and was purified by paper chromatography.

Enzymes

The method of Ballou and Luck (16) was used for the preparation of wheat B-amylase and that of Cori *et al.* (8) for phosphorylase. A crude preparation of maltase was obtained as described by Weidenhagen (17) and dialyzed.

Assay of the Glycogen-Forming Enzyme

The standard reaction mixture contained 0.23 μmole UDPG, 0.5 μmole G-6-P, 0.4 mg. glycogen, 3.75 μmoles Tris-maleate buffer of pH 8.5, 0.25 μmole EDTA, and enzyme, in a final volume of 0.05 ml. Incubations were carried out at 37º for 30 min. The reaction was stopped by heating for 1 min. in boiling water, and the UDP formed was measured. Under the conditions of the test, added UDP did not disappear on incubation with crude or purified muscle extracts.

The initial rate of reaction was calculated from a two or three-point time curve by applying the equation for first-order reactions and extrapolating the value of k to zero time

Preparation of the Enzyme

Rat muscle was used in most of the experiments. Only a small part of the activity was extracted with water from minced muscle. In order to obtain active extracts, it was necessary to homogenize the tissue thoroughly with a blendor. The extraction was usually carried out with water. With phosphate or pyrophosphate buffers the yield was slightly higher, but more inactive protein was extracted. The enzyme could be precipitated from the aqueous extracts either with 0.41 saturated ammonium sulfate or by adjusting the pH to 5.8-6.0.

The procedure which was used in most of the preparations was as follows. The muscles from two rats (about 55 g.) were cooled, minced, suspended in 3 vol. of cold water, and homogenized for 2-3 min. in a Waring blendor. The homogenate was imme-

diately centrifuged at 12,000 × *g* for 10 min. When this procedure was carried out quickly and at low temperature, the pH did not drop below 6.5

The supernatant crude extract was divided into two portions. One (about ¼) was heated at 100° for 5 min., and the precipitate was removed by centrifugation. The rest of the supernatant fluid (its volume is represented as *v* in he following) was acidified to pH 5.8-6.0 (chlorophenol red as indicator), and after 15 min. at 0° was centrifuged at 24,500 × *g* for 10 min. The precipitate was suspended in water, adjusted to pH 5.8-6.0, and centrifuged. The precipitate was then suspended in 0.2 *v* of heated extract and centrifuged again after freezing and thawing. The supernatant fluid, which was usually turbid and contained most of the activity, was acidified, centrifuged, and washed with water as previously. The precipitate ("second precipitate") was suspended in 0.05 *v* of 0.015 *M* Tris–maleate buffer of pH 7.4 containing 0.005 *M* EDTA.

The activity of these preparations decreased about 50% after storage overnight at - 15°. More stable preparations were obtained by lyophilizing the "second precipitate" and extracting with 0.05 *v* of 0.1 *M* pyrophosphate buffer of pH 8 containing 0.65 mM reduced glutathione. The latter preparations were completely colorless and transparent and were stable for at least a week stored at pH 7 and – 15°. The results obtained in the purification by the second procedure are shown in Table I. The purification obtained in that experiment was 12-fold with respect to protein, and the yield was about 10%. In other preparations the purification ranged from 20- to 40-fold.

Results

Stoichiometry

The stoichiometry of the reaction catalyzed by the glycogen-forming enzyme is shown in Table II. It may be observed that, in the complete system, for each mole UDPG that was utilized about 1 mole UDP was formed and 1 mole glucose was added to glycogen. In the absence of G-6-P or glycogen, the chemical changes were much smaller.

Effect of Increasing Enzyme Concentration

As shown in Fig. 1, increasing amounts of UDP were formed with increasing amounts of enzyme. It may be noted that for practical reasons the test system contained only 0.23 μmole UDPG, so that a linear relation was not to be expected. Furthermore, inhibition by UDP probably influences the course of the reaction.

TABLE II

Stoichiometry

Complete system as described in text, but with amounts doubled and 0.072 mg. glycogen.

	UDPG disappearance	UDP formed	Glycogen formed
	μmole	μmole glucose	μmole
Complete system	0.13	0.13	0.12
No G 6-P	0.05	0.03	0.05
No glycogen	0.015	0.02	—

TABLE I

Results of the Purification Procedure

Micromoles/hr./mg. protein

	Crude extract	Purified preparation	Ratio Purified/crude
UDP formation	1.4	17.5	12.5
Phosphorylase	75	34	0.45
Amylase (in glucose equivalents)	0.02	0.018	0.9
Protein concentration *(mg./ml.)*	38.7	8.2	—

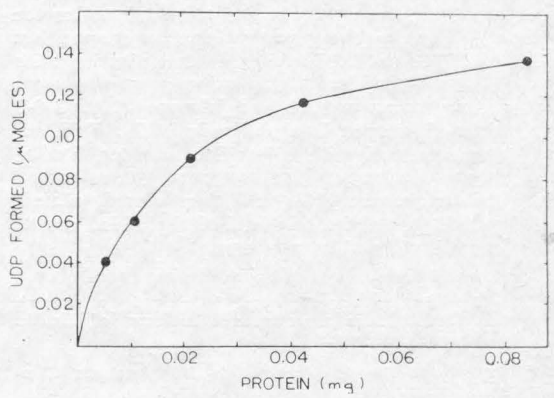

FIG. 1. The effect of enzyme concentration on the formation of UDP. Standard system as described in text.

Effect of UDPG *Concentration*

The course of the reaction with different UDPG concentrations is shown in Fig. 2. From these values, initial rates were calculated as described under Methods, and the method of Lineweaver and Burk (18) was applied. The value obtained for the Michaelis constant, which should be considered only approximate, was 5×10^{-4} *M*. It may be mentioned for comparison that the K_m for G-1-P in the phosphorylase reaction is $5.7 \times 10^{-3} M$ (19).

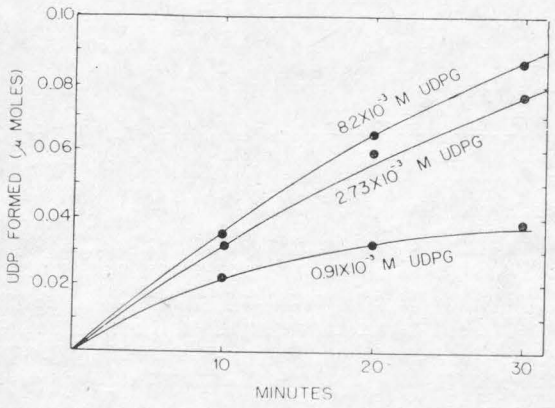

FIG. 2. Time curves with different amounts of UDPG. Standard system as described in text.

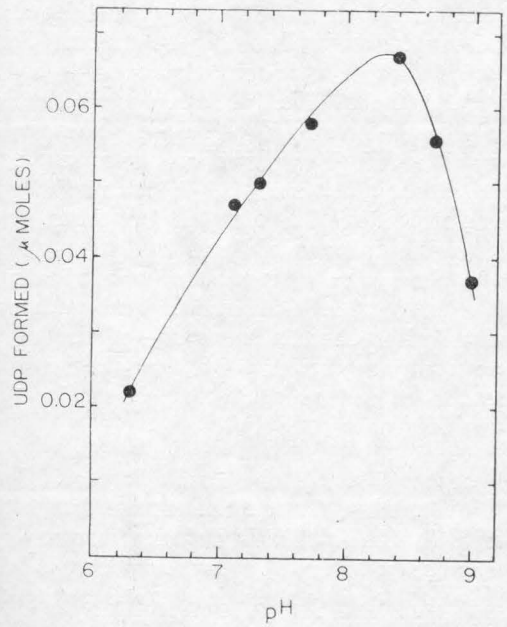

FIG. 3. pH –activity curve. Standard system as described in text, with Tris–maleate buffers of different pH. The final pH was checked on aliquots with a glass electrode. The solutions were neutralized before proceeding to the enzymic estimation of UDP.

pH Optimum

As shown in Fig. 3, the pH for maximal activity in Tris-maleate buffer was 8.3.

Primer Requirements

Figure 4 shows the effect of glycogen concentration on the rate of UDP formation from UDPG. The results of experiments employing different polysaccharides as primers are shown in Table III. Application of a first-order equation to such results gave values which changed greatly with time so that the percentages given in Table III were calculated from single time values and are probably not strictly proportional to priming ability. It may be observed that the primer requirements of the glycogen-forming enzyme are not very different from those of muscle phosphorylase. That is, the best primer is glycogen and its activity decreases after degradation. A more detailed study will be required to determine whether the differences observable in Table III are in fact real.

Substances which failed to give any detectable stimulation when tested as primers were: glucose, mannose, maltose, lactose, cellobiose, trehalose, raffinose, melibiose, and gentiobiose. Commercial dextrin (Difco) produced a slight activation.

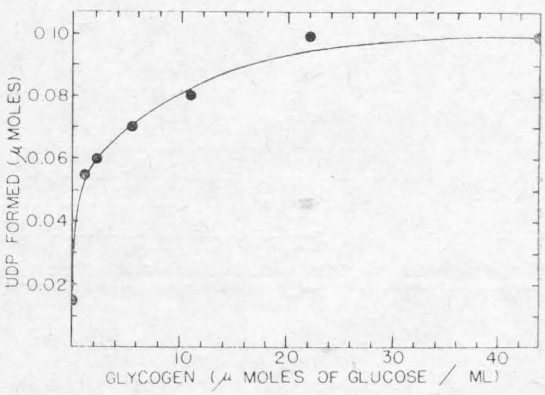

FIG. 4. The effect of varying glycogen concentration on enzyme activity. Standard system as described in text.

TABLE III

Activating Effect of Different Polysaccharides

The results are expressed in per cent activation in relation to glycogen. The final concentration of polysaccharide was 5.4 mg./ml. in every case. Such a concentration of glycogen was just enough to produce maximal activity. All activities were tested with the same enzyme preparation. Numbers in brackets represent a second experiment. Incubation time: 30 min.

Polysaccharide	UDP formation from UDPG	Pi formation from G-1-P
Glycogen	100	100
Phosphorylase limit dextrin from glycogen[a]	59 (52)	28 (20)
β-Amylase limit dextrin from glycogen[a]	30 (31)	15 (20)
Glycogen treated with α-amylase	0 —	6 —
Commercial soluble starch (blue with iodine)	17 (20)	5 (10)
Soluble starch[b] (red with iodine)	19 (13)	0 (0)
Potato starch (heated in alkali)	59 (48)	9 (20)

a Prepared as described by Hestrin (20).

b Obtained by treatment with acid according to Lintner (21).

Activators and Inhibitors

Phloridzin at $3.3 \times 10^{-3} M$ concentration inhibited 48 %. At the same concentration the inhibition of phosphorylase was 42 %. These values were obtained from the change in initial rates calculated as described under *Methods*. With glucose at 0.05 *M* concentration, the inhibition was 52 %, as compared to 72 % for phosphorylase. The effect of added UDP was tested both by the standard method and by measuring glycogen formation. At $10^{-3} M$ of added UDP, the inhibition was 31 %; and at $5 \times 10^{-4} M$ it was 18 %.

The reaction was inhibited 80 % by 0.2 *M* potassium borate and 58 % by 0.1 *M* KCN. Two antidiabetic substances 1-*p*-tolysulfonyl-3-butylurea and 1-(*p*-aminobenzenesulfonyl)-3-butylurea (0.2 % final concentration) inhibited slightly (15 and 8 %, respectively). Cysteine or reduced glutathione produced a slight activation on some enzyme preparations. The following substances did not change the activity: Mg^{++}, G-1-P, adenosine triphosphate, adenosine 5'-monophosphate, galactose 1-phosphate, and insulin.

The Action of Glucose 6-*Phosphate*

Partially purified preparations of the glycogen-forming enzyme were found to be activated by heated extracts. Heated pigeon muscle extracts were particularly active. The active substance proved to be stable in acid, labile in alkali, and behaved as an acid when treated with anion-exchange resins. Many known substances were tested as possible substitutes for the heated extract. Of these G-6-P and fructose 6-phosphate proved to be active. Since the preparation contained an active phosphoglucoisomerase, it could not be decided which of the two produces the activation. A sample of G-6-P obtained by chemical synthesis was found to be active. Many other substances were tested as possible substitutes for G-6-P. The following were ineffective: glucose, maltose, trehalose, fructose 1-phosphate, trehalose phosphate, sucrose phosphate, lactose, citrate, G-1-P, adenosine triphosphate, adenosine 5'-monophosphate, glutamic acid, inorganic phosphate, and galactose 1-phosphate. Activation was obtained with glucosamine 6-phosphate and with galactose 6-phosphate. The sample of the latter was contaminated with G-6-P, but the amount appeared to be too small to account for the activation.

A curve showing the formation of UDP with different G-6-P concentrations is shown in Fig. 5. Half-maximal velocity was attained at about $6 \times 10^{-4} M$ concentrations of G-6-P. In order to obtain information on the mechanism by which G-6-P increases the rate of reaction, some experiments were carried out with labeled compounds. As shown in Table IV, while the radioactivity of C^{14} UDPG was recovered in the glycogen, that of

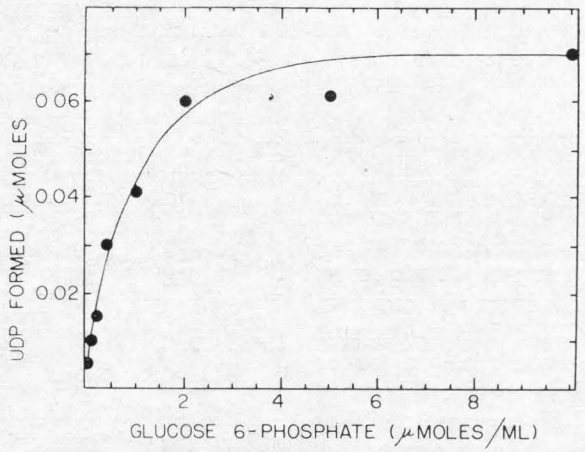

FIG. 5. — The effect of changing G-6-P concentration. Standard system as described in text.

TABLE IV

Incorporation of Radioactivity into the Glycogen

Complete system as described in text except for the additions indicated and that the amount of glycogen was increased to 1.2 mg.

The reaction was stopped by adding 0.9 ml. of 33 % KOH. After heating 20 min. at 100°, the glycogen was precipitated with 1.25 ml. ethanol, boiled, centrifuged, reprecipitated, and plated for counting the radioactivity.

Additions				Counts/min. in glycogen
UDPG		G-6-P		
μ *mole*	*counts/min.*	μ *mole*	counts/min.	
0.16	4,950	0.80	0	2,550
0.16[a]	4,950[a]	0.80	0	0
0.25	0	0.43	51,500	120
0.25[a]	0[a]	0.43	51,500	30

[a] Added after incubation.

TABLE V

Nondisappearance of G 6-P

Standard system as described under *Methods* except for the G-6-P concentration.

Incubation time	G-6-P estimation	UDP formed
min.	μ *mole*	μ *mole*
Complete system 0	0.097	0
Complete system 30	0.098	0.057
No glycogen 30	0.099	0.003

C^{14} G-6-P was not. Moreover, estimations of G-6-P + fructose 6-phosphate showed that there was no measurable change in concentration during the reaction (Table V).

The assumption that hexose phosphate might act as primary acceptor of the glucose residue and that a disaccharide phosphate would then serve as donor to the glycogen, was examined by several methods. One was to add phosphopyruvate and pyruvate kinase during the reaction in order to favor the first step by removing the UDP. Even under these conditions the reaction required a polysaccharide primer, showing that the first step would not take place without the second, or that only one step is involved in the overall reaction. Other experiments consisted in adding disaccharide phosphates such as sucrose or trehalose phosphate instead of UDPG and testing for increase in glycogen. The results were negative.

Radioactive Glycogen

Incubation of C^{14} UDPG led to the labeling of the glycogen. In a typical experiment 1.2 μmoles of C^{14} UDPG (12,400 counts/min.) were incubated with an eightfold dose of the standard mixture containing 4.8 mg. glycogen. After 30 min. at 37° the mixture was boiled in alkali, precipitated with 1.25 vol. ethanol, and reprecipitated. An aliquot was plated for measuring radioactivity. The number of counts per minute in the glycogen fraction for the total sample was 10,000. That is, the incorporation reached about 80 %. It may be mentioned that the C^{14} UDPG preparation contained about 25 % UDP-galactose, so that the incorporation obtained

was complete within experimental errors. Controls in which the reaction was stopped at $t = 0$ gave no radioactivity.

Reaction Products

The method used for the estimation of UDP is not very specific. Other nucleoside diphosphates and also adenosine monophosphate are active in this test system. However, uridine monophosphate is inert. In order to check the formation of UDP in the enzymic reaction, the products were run on paper with an ethanol-ammonium acetate solvent of pH 7.5 (22). A spot with the same mobility as UDP was observed in the zone corresponding to the complete sample, but not in that of controls where the reaction was stopped at zero time or incubated without UDPG. Furthermore, it may be mentioned that no inorganic phosphate was found to be liberated in the reaction, and that no formation of oligosaccharides could be detected by paper chromatography.

As to the characterization of the polysaccharide formed in the reaction, the usual methods could not be used because only small amounts of UDPG were available. Therefore enzymic degradation and paper chromatography were utilized. The procedure consisted in isolating the glycogen formed from C^{14} UDPG as previously described. The radioactive glycogen obtained was treated with β-amylase which is known to hydrolyze alternate $1 \rightarrow 4$ α-linkages yielding maltose. The reaction mixture was then treated with 3 vol. methanol. The soluble portion which contained all the radioactivity was run on paper with the pyridine-butanol-water solvent (6:4:3) (23). As shown in Fig. 6*A*, the product obtained behaved like maltose with a trace of glucose. All the radioactivity was found in the disaccharide spot. Since the afore-mentioned solvent did not separate the disaccharides, a sample which had been chromatographed was wetted with borate buffer and submitted to electrophoresis. As shown in Fig. 6*B*, the radioactivity was found in a spot having the same mobility as maltose. Electrophoresis was carried out after paper chromatography because in that manner the interference of salts and residues of protein was obviated.

As an additional test, the product obtained by the action of β-amylase was treated with a maltase-containing yeast extract. As shown in Fig. 7, the radioactivity appeared in a spot migrating like glucose.

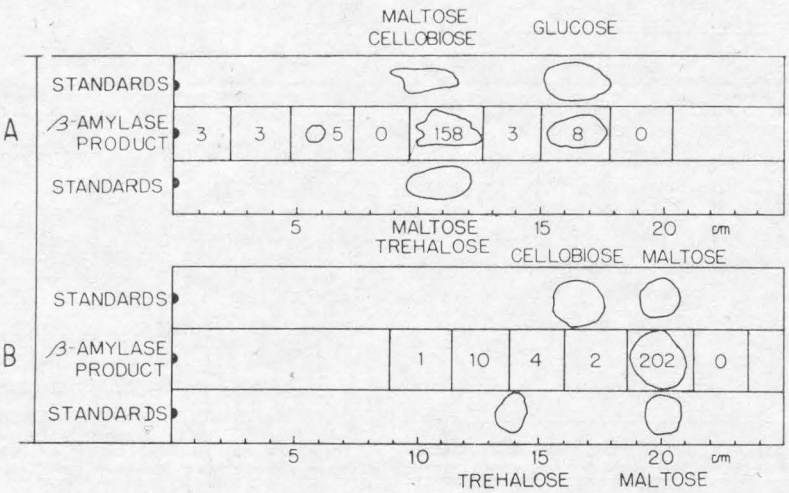

FIG. 6. — *A*: Paper chromatography of the product obtained by the action of β-amylase on the glycogen formed from radioactive UDPG. The glycogen was obtained as described in text. An aliquot was treated with β-amylase at pH 6 (20) for 20 hr. at 37°. Three volumes of methanol was added. The supernatant which contained all the radioactivity was spotted on paper and run with the butanol-pyridine-water solvent. The radioactivity of the sugar spots was then detected as described in text. The squares in the graph show how the papers were cut and the numbers represent counts per minute after substracting background.

B: Same as *A*, but after chromatography the paper was wetted with 0.05 *M* borate buffer of pH 9 and submitted to electrophoresis at 600 v. for 6 hr.

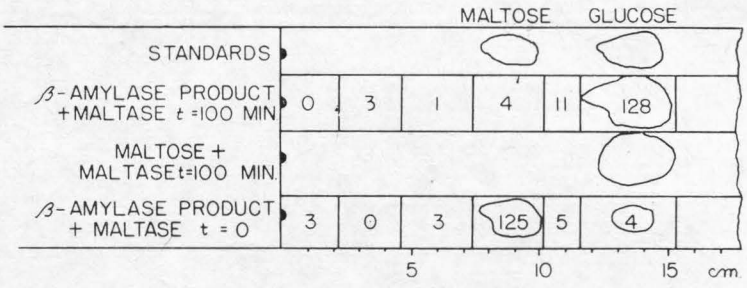

FIG. 7. — Paper chromatography of the product obtained by the action of β-amylase on radioactive glycogen submitted to the subsequent acion of a maltase containing yeast extract. General procedure as in Fig. 5.

In other experiments the radioactive glycogen was degraded with phosphorylase and excess inorganic phosphate (20). On precipitation with 3 vol. methanol, no radioactivity remained in the precipitate containing the limit dextrin. The soluble portion was freed from methanol by exaporation and separated into two fractions by $ZnSO_4$ and $Ba(OH)_2$ precipitation. The precipitate containing G-1-P was hydrolyzed with acid (24), and the free sugar formed was chromatographed on paper with the butanol-pyridine-water solvent. Radioactivity was found only in the glucose spot.

Distribution

The activity of extracts of different rat organs is shown in Table VI. It may be observed that more activity is found in muscle, heart, and liver than in the other organs. Positive results were also obtained with pigeon liver and muscle and with rabbit muscle.

DISCUSSION

There are some facts which indicate that the classical scheme for glycogen synthesis may not be correct since it implies the action of phosphorylase, and the ratio of inorganic phosphate to G-1-P as measured in whole tissues is usually too high for net synthesis to take place (25). Furthermore, agents which cause glycogen degradation produce a concomitant increase in phosphorylase concentration. Thus the glycogenolytic action of epinephrine (26, 27) glucagon, and high Na^+ ions (28) has been attributed to an increase in phosphorylase activity. Other facts have also been interpreted as meaning that phosphorylase functions mainly in the degradation of glycogen (25, 29). If these conclusions are correct, then some other mechanism or mechanisms should be involved in glycogen synthesis and the enzyme described in this paper might well serve this purpose. In the intact rat liver the rate of glycogen formation is about 30 μmoles/hr./g. (30) and, as shown in Table VI, liver extracts under optimal conditions can catalyze the transfer of about 190 μmoles glucose from UDPG/hr./g. tissue. The values for muscle are 5-17 (31) and 220, respectively. In the whole organ the enzyme is, presumably, not working at saturating concentrations of substrate and activator so that the two values might be in fair agreement.

TABLE VI

Distribution in Different Rat Organs

Results expressed as micromoles UDP formed/hr./g. tissue. Organs were homogenized in 3 vol. water.

Muscle	220	Spleen	37
Heart	166	Kidney	31
Liver	187	Lung	36
Brain	32		

It may be mentioned that tissues have much more phosphorylase than the glycogen-forming enzyne; thus when the rate of glycogen formation from UDPG and from G-1-P was compared in crude muscle extracts under optimal conditions and in the presence of activators (G-6-P and adenylic acid, respectively), it was found that the latter process (phosphorylase reaction) was 20-50 times faster.

The tests which have been carried out on the radioactive glycogen formed from C^{14} UDPG indicate that the glucose residue becomes attached by α $(1 \rightarrow 4)$ linkage. This is

the same type of linkage which is hydrolyzed by phosphorylase.

As to the mechanism by which G-6-P increases the activity of the glycogen-forming enzyme, no clue was obtained. The effect was also obtained with fructose 6-phosphate, glucosamine 6-phosphate, and galactose 6-phosphate. No indication in favor of the intermediary formation of a disaccharide phosphate could be shown.

SUMMARY

An enzyme which leads to the formation of glycogen according to the equation:

UDPG + primer → UDP + glucosyl $\alpha(1 \rightarrow 4)$ primer

has been studied.

The optimal conditions for activity were determined with a partially purified preparation from rat muscle. The reaction requires the presence of a polysaccharide as primer and is strongly activated by hexose 6-phosphates. Using UDPG labeled in the glucose moiety, it was found that the radioactivity was transferred to the glycogen from which it could be removed as maltose with β-amylase or as G-1-P with phosphorylase. Thus it seems that the glucose residue becomes linked α $(1 \rightarrow 4)$ to the polysaccharide. Several inhibitors were tested as well as the occurrence of the enzyme in different organs.

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NATURE
Vol. 187, p. 918, 1960

MECHANISM OF STARCH BIOSYNTHESIS

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Since the classical work of Hanes[1] some workers consider that starch synthesis *in vivo* is catalysed by phosphorylase. Nevertheless, others have raised doubts on this hypothesis. Thus Ewart *et al.*[2], from measurements of the ratio of inorganic phosphate to glucose-1-phosphate, concluded that: "phosphorylase is not involved in the synthesis of starch... but the role of phosphorylase in the normal metabolic breakdown... is not questioned" (cf. also Rowan and Turner[3]). Furthermore, Stocking[4] reported that starch synthesis in leaves is initiated in the chloroplasts, where phosphorylase could not be detected.

The problem of starch synthesis is similar to that of glycogen synthesis in animal tissues. The synthetic role of phosphorylase *in vivo* has been challenged on the basis of the following facts: a) the unfavourable ratio of inorganic phosphate to glucose-1-phosphate in tissues; b) agents which increase the concentration of phosphorylase, such as epinephrine and glucagon, produce glycogen breakdown[5]; and (c) in certain diseases phosphorylase is absent in the muscles, although normal or increased amounts of glycogen are present[6].

If these facts are sufficient to rule out phosphorylase as the enzyme responsible for glycogen synthesis, the only enzyme which can take its place, so far as we know, is glycogen synthetase[7], which catalyses the transfer of glucose from uridine diphosphate glucose to glycogen forming a new α-1:4 linkage. On the other hand, it is known that uridine diphosphate glucose acts as glucose donor in plants. Thus enzymes catalysing the synthesis of sucrose[8], sucrose phosphate[9], and callose[10] have been described. The latter is a β-1:3 glucan[11] discovered many years ago and believed to have a role in the physiology of sieve tubes and in wound reaction in plants[12].

All this suggests that uridine diphosphate glucose should be involved in starch synthesis. Although incubation of radioactive uridine diphosphate glucose with different plant materials usually resulted in the incorporation of radioactivity in the polysaccharide fraction, the product formed was insoluble in hot water, was not hydrolysed by β-amylase and was presumably callose.

Since glycogen synthetase has been found to be strongly adsorbed on glycogen (ref. 7*c*), we have looked for a starch-synthesizing enzyme in starch granules. In preliminary experiments, radioactive uridine diphosphate glucose was incubated with freshly prepared potato starch and it was found that there was some incorporation of radioactivity in the fraction which became soluble by the subsequent action of α-amylase. More active preparations were obtained from beans as follows. Freshly harvested immature dwarf string beans (*Phaseolus vulgaris* var. Bountiful) were used. The cotyledons and the embryos were ground in a mortar with two volumes of water. The suspension was filtered through cheesecloth and centrifuged 5 min. at 3,000 rev./min. The precipitate was resuspended in several volumes of water and recentrifuged three times. The white precipitate was then suspended in four volumes of acetone at — — 15°C., centrifuged at 0°C.; this procedure was repeated three times, after which the precipitate was dried *in vacuo*. This preparation could be stored for months at — 15° C. with no decrease in activity.

The bean starch fraction (2 mgm. containing 6 μgm. of protein) was incubated at 37° C. for 3 hr. with 0.21 μmole of uridine diphosphate glucose containing 7,850 counts/

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min. of carbon-14 in the glucose moiety, 2 μmoles of glycine buffer of *p*H 8.4, 0.05 μmole of ethylenediamine tetraacetate in a final volume of 15 μl. After incubation, 0.5 ml. of 80 per cent ethanol was added. The soluble fraction was spotted on paper and chromatographed with ethanol-ammonium acetate of *p*H 7.5 (ref. 15). The uridine diphosphate and uridine diphosphate glucose spots were eluted from the paper and the absorbancy at 260 mμ, radioactivity and uridine diphosphate were measured. The insoluble fraction was washed with ethanol and counted. Blanks without and with uridine diphosphate glucose added after incubation were run at the same time. Furthermore, it was found that the bean starch fraction did not destroy added uridine diphosphate.

The changes occurring on incubation of the bean starch fraction with radioactive uridine diphosphate glucose are shown in Table 1; these results indicate a correspondence between disappearance of uridine diphosphate glucose, formation of uridine diphospate and incorporation of glucose into the starch fraction.

The identity of the product formed was investigated as follows. The starch fraction after incubation with radioactive uridine diphosphate glucose was thoroughly washed with aqueous ethanol, suspended in water, heated for 10 min. at 100°C. and then treated with β-amylase. Wheat β amylase was allowed to act for 12 hr. at 37° C., and 3 volumes of methanol were added. The soluble fraction was evaporated, spotted on paper and

TABLE I

Analysis of substrates and products

	μmole	μmole
Disappearance of uridine diphosphate glucose	0.02 [1]	0.016 [2]
Formation of uridine diphosphate	0.017 [1]	0.015 [3]
Glucose-U-14C incorporated into starch		0.017 [3]

1 Calculated from absorbancy at 260 mμ
2 Calculated from radioactivity.
3 Estimated with pyruvate kinase (ref. 7*c*).

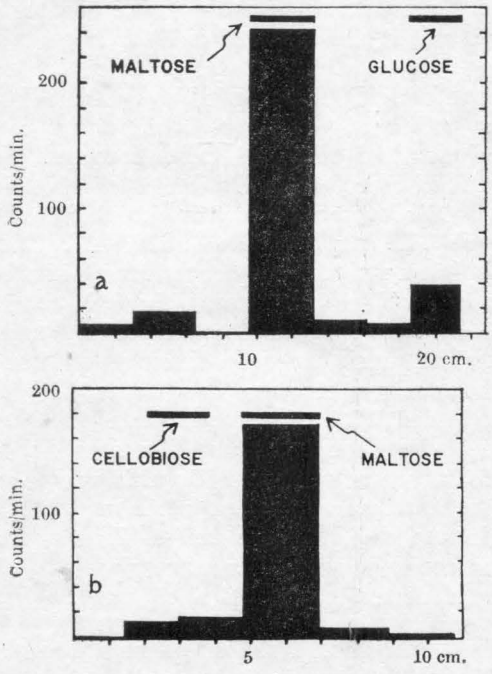

FIG. 1. — (a) Paper chromatography; and (b) paper electrophoresis of reaction product after treatment with β-amylase.

chromatographed with butanol/pyridine/water (6:4:3 v/v) [13] as solvent. As shown in Fig. 1*a* the radioactivity was found to migrate like maltose. For further confirmation of the identity of the substance, the 'maltose' eluted from the paper after chromatography was submitted to electrophoresis with borate buffer [14]. As shown in Fig. 1*b*, the radioactivity migrated with the 'maltose'. Under these conditions, cellobiose, laminaribiose and gentiobiose are neatly separated from maltose.

TABLE 2

Donor specificity

Bean starch fraction (8 mgm.) incubated 1 hr. under conditions similar to those indicated in Table I with about 0.2 mole of substrates indicated.

Donor	Counts/min. added	Counts/min. recovered in starch fraction
Uridine diphosphate glucose	5,300	590
Glucose-1-phosphate	8,900	190
Glucose-6-phosphate	10,000	210
Sucrose	8,500	130
Glucose	10,000	0

Similar results were obtained with α-amylase. Saliva (0.1 volume) was allowed to act for 30 min. at 37° C. and the samples processed as described for β-amylase. The results were similar except that radioactivity appeared also in the maltotriose spot.

These results are consistent with the following formulation:

uridine diphosphate glucose + acceptor → uridine diphosphate + α-glucosyl-1,4-acceptor (1)

in which the acceptor is starch.

Other experiments were carried out in order to study the specificity of uridine diphosphate glucose as glucose donor. As shown in Table 2, glucose phosphates and sucrose showed some incorporation of radioactivity but much less than uridine diphosphate glucose.

Sucrose has been tested as glucose donor in other experiments with various plant tissue preparations with negative results. The well-known ready conversion of sucrose into starch *in vivo* might take place indirectly with uridine diphosphate glucose as an intermediate. The formation of uridine diphosphate glucose from sucrose has been detected (refs. 8, 9) and may be written as follows:

sucrose + uridine diphosphate ⇌ uridine diphosphate glucose + fructose (2)

The addition of reactions (1) and (2) would result in the formation of starch from sucrose.

This investigation was supported in part by a research grant (Nº G-3442) from the National Institutes of Health, U.S. Public Health Service and by the Consejo Nacional de Investigaciones Científicas y Técnicas.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 236, p. 636, 1961

STARCH AND OLIGOSACCHARIDE SYNTHESIS FROM URIDINE DIPHOSPHATE GLUCOSE *

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Several reactions in which uridine diphosphate glucose acts as a glucose donor have been described. The products of these reactions are: trehalose phosphate (1), sucrose (2), sucrose phosphate (3), bacterial cellulose (4), callose (5), glucosides (6), and glycogen (7-9). In a previous paper (10), it was reported that the starch granule fraction of plant tissue catalyzes the incorporation of the glucose moiety of uridine diphosphate glucose into starch. The results of enzymic degradation of the reaction product were consistent with the following formulation:

$$\text{UDP-glucose} + \text{acceptor} \rightarrow \text{UDP} + \alpha\ (1 \rightarrow 4)\ \text{glucosyl-acceptor}$$

Furthermore, it was reported that sucrose, glucose 1-prosphate, and glucose 6-phosphate were either ineffective or inferior to uridine diphosphate glucose as glucose donors. Further work on the subject is reported in this paper.

EXPERIMENTAL PROCEDEURE

Substrates — UDP-glucose was obtained according to Pontis *et al.* (11). Radiactive UDP-glucose was prepared by incubation of glucose-6-P-C^{14} with UDP-glucose and *Saccharomyces fragilis* extract and isolated by paper chromatography (12). Malto-oligosaccharides were prepared by hydrolysis of amylose (13) and separation by a charcoal-Celite column (14). Radioactive samples of these oligosaccharides labeled at the reducing glucose unit were obtained from glucose-C^{14} with D-enzyme and soluble starch (15) and separated by paper chromatography.

Preparation of Enzyme — Active extracts have been obtained from young patotoes, sweet corn, and beans. A preparation of the latter was used in all the experiments described in this paper. The procedure was as follows. Freshly harvested immature dwarf beans (*Phaseolus vulgaris* Bounfitul) were peeled, and the cotyledons and embryos were ground in a mortar with 2 volumes of water. The coarse material was removed by straining through cheese cloth, and the suspension of starch granules was centrifuged for 5 minutes at 3000 r.p.m. The white sediment was suspended in several volumes of water and recentrifuged. After the procedure had been repeated three times, the starch granules were suspended in 4 volumes of acetone at −15° and centrifuged at 0°. The latter procedure was repeated three times, after which the starch granules were dried in a vacuum.

Measurement of Enzyme Activity — The standard reaction mixture, unless otherwise indicated, contained (in μmoles): UDP-glucose, 0.3; EDTA,[1] 0.1; glycine buffer at pH 8.4, 4; and 5 mg of enzyme preparation. The total volume of fluid was 14 μl. After incubation at 37°, UDP formation, radioactivity, or both, were measured as follows.

UDP Formation — The pyruvate kinase procedure described previously was used (9). When the bean enzyme preparation was heated in order to stop the reaction, starch paste was formed which made difficult the

* This investigation was supported in part by a research grant (No. G-3442) from the National Institutes of Health, United States Public Health Service, by the Rockefeller Foundation and by he Consejo Nacional de Investigaciones Científicas y Técnicas.

† This work was carried out during the tenure of a postdoctoral fellowship from the Univtrsity of Buenos Aires.

1 The abbreviation used is: EDTA, ethylenediaminetetraacetate.

subsequent mixing with the reagents. Therefore, in most experiments, the phosphopyruvate and pyruvate-kinase were added without inactivating the starch-forming enzyme.

Radioactivity Measurements — (*a*) *Starch*: After enzyme action, 0.5 ml of 50 % esthanol was added and the suspension was centrifuged. The supernatant fluid containing oligosaccharides was set aside. The white precipitate was washed four times with 0.5 ml of 50 % ethanol and then suspended in 0.4 to 0.5 ml of water, heated for 10 minutes of 100° in order to disperse the starch. Suitable aliquots were then plated on aluminum disks and counted with a gas flow counter (Tracerlab, Inc.). No correction was applied for self-absorption.

In experiments in wich oligosaccharides were formed, it was observed that they were not removed completely from the starch by the above mentioned procedure. Therefore, in some experiments, the starch granules were first ruptured by heating in 0.2 ml of 0.1 % Na_2SO_4 during 10 minutes at 100°, 2 volumes of 95 % ethanol were added, and the suspension was centrifuged. The supernatant fluid was used for oligosaccharide estimation. The precipitate was redissolved in dilute Na_2SO_4 as before and reprecipitated. After the procedure was repeated four times, and aliquot was plated for measuring radioactivity.

(*b*) *Oligosaccharides*: The supernatant fluid obtained after the starch was centrifuged off as described above was diluted with 1 volume of water and passed through a column 0.5 cm diameter × 5 cm long of mixed-bed resin (Amberlite MB 3, acetate). The percolate was concentrated and either plated on aluminuim disks for measuring the radioactivity or spotted on paper for chromatography. The solvent used was butanol-pyridine-water (6:4:3) (16), and the paper used was Whatman No. 1 or 4. The times of development were, respectively, about 48 and 16 hours. Known samples of oligosaccharides were run at the same time and located with silver-NaOH (17). The radioactivity was measured (*a*) by cutting 1-cm strips, eluting with water, and plating on aluminium disks, (*b*) by introducing the paper strips directly in a gas flow counter, (*c*) by scanning automatically with a Nuclear-Chicago model D-47 gas flow counter fitted to C-100A actigraph II, (½ in collimator). The relative number of counts obtained in procedures *a*, *b*, and *c* were, respectively, about 100, 40, and 30.

Separation of Starch Components — The starch (5 mg) was defatted by washing three times with 1 ml of hot methanol and then was suspended in 1 ml of water. The suspension was heated at 100° and homogenized intermittently in a tightly fitting small glass homogenizer for 1 hour. A small precipitate was removed by centrifugation, and 0.01 ml of 10 % thymol in ethanol (18) was added. After 3 days at room temperature, the precipitate (amylose fraction) was separated from the supernatant (amylopectin fraction).

Bromine Oxidation — The oligosaccharides separated by paper chromatography were eluted with water. An aliquot (0.5 ml) was treated with Br_2 and $BaCO_3$ as described by Smith and Srivastava (19). Excess Br_2 was removed by aeration, the solution was passed through a cation exchange resin (Dawex 50-H^+), and hydrolysis was carried out in 1 N HCl at 100° for 1 hour. The solution was concentrated under reduced pressure and dried in a desiccator over NaOH. After the residue was redissolved and neutralized with KOH, paper chromatography was carried out with butanol-pyridine-water (16) as solvent. The zones corresponding to glucose and gluconic acid were then counted directly on the paper with a gas flow counter.

Borohydride Reduction — The samples obtained as for bromine oxidation were treated with borohydride as described by Walker and Whelan (15), hydrolyzed, and chromatographed on paper with ethyl-acetate-boric acid-acetic acid as solvent. The sorbitol and glucose zones were then counted as described before. In this procedure, as with Br_2 oxidation, the losses were considerable when small amounts (about 1 μmole) of oligosaccharides were used. In every case, known samples of labeled oligosaccharides were run at the same time.

Results

Properties of Enzyme — The dried enzyme preparations could be stored for several months at −15° without appreciable loss of activity. Grinding the enzyme in the dry state or in buffer solutions led to complete inactivation. The dry enzyme resisted heating for 5 minu-

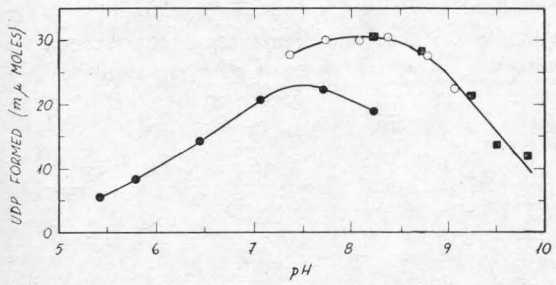

FIG. 1. — pH optimum. ○ glycyl-glycine; ■ glycine, ● phosphate buffer. The enzyme preparation (2 mg) was incubated for 2.5 hours (in μmoles) with: UDP-glucose, 0.32; EDTA, 0.1; and buffer indicated, 2. Total volume was 14 μl. Before UDP was measured, 4 μmoles of glycine, buffer of pH 8.4 were added in order to correct for any change of pH that might affect the pyruvate kinase reaction.

tes at 100°. Suspended in water, about 25 % of the activity was lost in 5 minutes at 50° and about 80 % at 60°. Many attempts to extract the enzyme from the starch granules with different buffers, digitonin, or detergents gave negative results. The addition of EDTA (0.01 M) did not affect the activity and neither did 0.01 M Mg++. The pH optimum was found to be about 8.2 in glycine or glycyl-glycine buffer and about 7.5 in phosphate buffer (Fig. 1). The rate of reaction increased with increasing amounts of enzyme (Fig. 2), but the response was not quite linear. The activity of the enzyme increased with temperatures up to 45° (Fig. 3), at which temperature the rate of reaction fell with time. At 37°, the reaction followed a linear course up to at least 4 hours, when the starch granules were not allowed to sediment either by shaking or by keeping the fluid volume low in relation to the amount of solids. Thus, with 15 μl of liquid for 5 mg of dry enzyme preparation, no visible sedimentation occurred during incubation.

The K_m for UDP-glucose was found to be about 6×10^{-2} (Fig. 4).

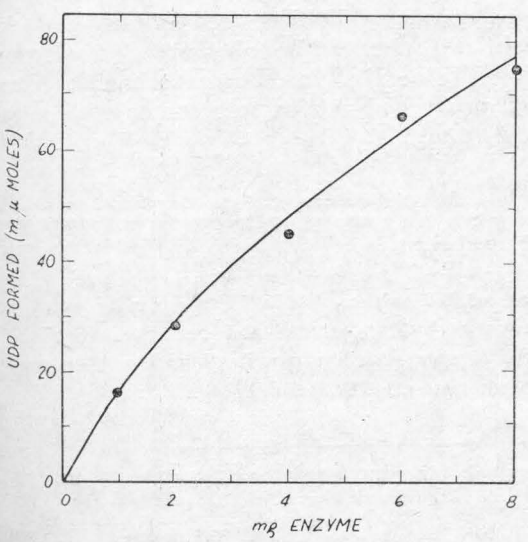

FIG. 2. — Proportionality between action and amount of enzyme. The amounts of enzyme preparation indicated were incubated 1.5 hours at 37° with the standard reaction mixture.

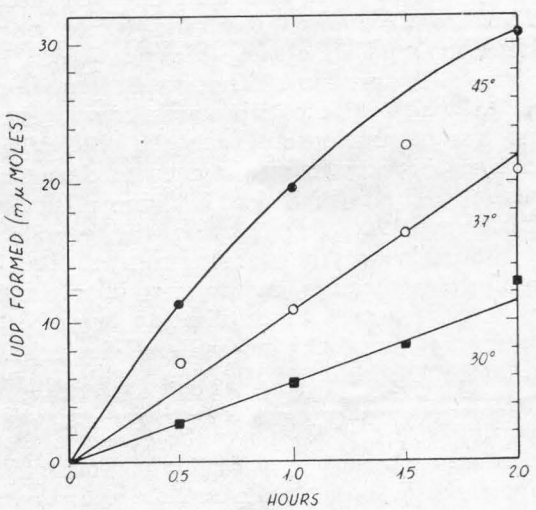

FIG. 3. — Effect of temperature. The enzyme preparation (2 mg) was incubated (in μmoles) with; UDP-glucose, 0.32; glycine, pH 8.2; 2; and EDTA, 0.05. Total volume was 16 μl.

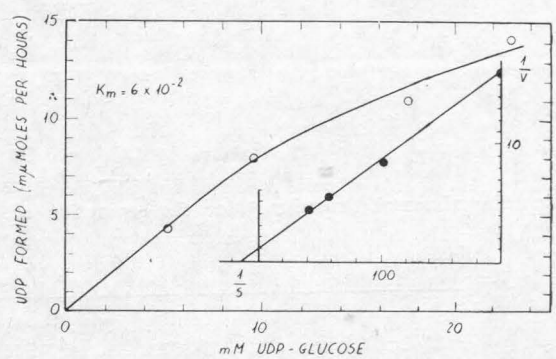

FIG. 4. — Effect of UDP-glucose concentration. The standard reaction mixture with 2 mg of enzyme and variable amounts of UDP-glucose was incubated. The rates were calculated from straight lines joining the results of 1-, 2-, and 3-hour incubation periods.

The main component of the enzyme preparation is starch. The protein content was found to be 3 μg per mg when measured with the method of Lowry *et al.* (20) after heating the starch granules 10 minutes at 100°. If the heating was omitted, the results were about 50 % lower.

Separation of Amylose and Amylopectin — In the previous paper (10), it was reported that after incubation of UDP-glucose-C^{14} with the enzyme preparation, the radioactivity was transferred to starch and could be recovered as maltose by treatment with α-or β-amylase. These results showed that the glucose transferred from UDP-glucose becomes joined in α (1 — 4) linkage but did not give information as to which of the two starch components, amylose or amylopectin, was the glucose acceptor. An experiment designed to clear this point is shown in Table I. Some difficulty was experienced in achieving the complete solubilization of the starch granules. By simultaneous heating and mixing in a small glass homogenizer, solubilization was fairly good, and practically no radioactivity remained in the insoluble fraction. Subsequent separation with thymol gave the results appearing in Table I. It may be observed that the approximately equal amounts of radioactivity were found in the amylose and amylopectin fractions. The specific activity was about 3-fold higher in the former. However, it does not seem that any clear-cut conclusion can be drawn from these experiments, as to whether amylose is the precursor of amylopectin, or *vice versa,* or if both act as direct glucose acceptors from UDP-glucose.

Glucose Transfer to Oligosaccharides — It was found that the bean enzyme preparation catalyzed the transfer of glucose from UDP-glucose to malto-oligosaccharides. The process was studied in two ways, (*a*) with labeled UDP-glucose and unlabeled oligosaccharides, (*b*) with labeled oligosaccharides and unlabeled UDP-glucose.

(*a*) *Labeled UDP-glucose* — As shown in Fig. 5, the addition of maltose or maltotriose to the enzyme and UDP-glucose-C^{14} was found to lead to the formation of substances which migrated on paper as the corresponding higher oligosaccharides. Thus, addition of maltose gave mainly maltotriose and some tetraose and pentaose. Likewise, addition of maltotriose led to the formation of mainly tetraose and some pentaose and hexaose.

In other experiments, the radioactivity in the total oligosaccharides was measured by first removing the starch by precipitation with ethanol and the UDP-glucose-C^{14} with an ion exchange resin. One such experiment is shown in Table II. Addition of maltotriose to the enzyme and UDP-glucose-C^{14} increased UDP, led to the appearance of oligosaccharides, and decreased incorporation of radioactivity into starch. Glucose also decreased starch formation, but without giving rise to oligosaccharides. Other saccharides such as fructose, sucrose, cellobiose, gentiobiose, and salicin did not lead to oligosaccharide formation (Table II). In other experiments, glycogen was tested as glucose acceptor with negative results.

TABLE I

Separation of starch components

The standard reaction mixture, containing 0.32 μmole of UDP-glucose-C^{14} (17,000 c.p.m.), was incubated for 3 hours at 37°. The starch granules were washed with 50 % ethanol and treated as described in "Experimental Procedure" for the separation of starch components. The two fractions were then analyzed for radioactivity and glucose content (21).

	Radioactivity	Glucose content	Specific activity
	c.p.m.	*μmoles*	*c.p.m. / μmole of glucose*
Amylose	1100	6.6	166
Amylopectin	980	19.0	51

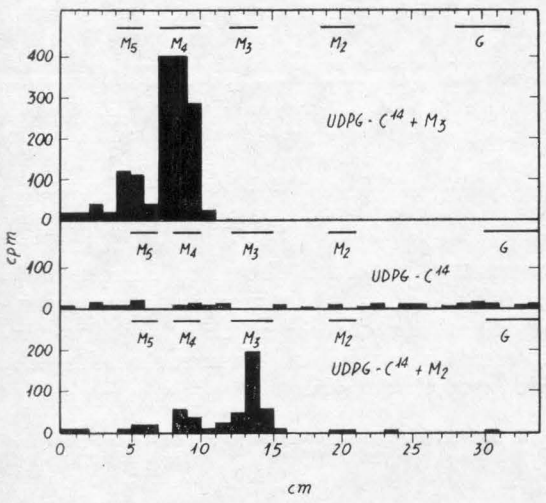

FIG. 5. — Labeled UDP-glucose and unlabeled maltose and maltotriose. The standard reaction mixture (in μmoles) with: 0.22 of UDP-glucose-C14 (12,000 c.p.m.) plus 5 of maltose or 1 of maltotriose was used. The incubation lasted 1.5 hours except the maltotriose samples which were incubated 3 hours. After paper chromatography, the different zones were eluted and counted. The abbreviations used are: M_2, maltose; M_3, maltotriose; M_4, maltotetraose and M_5, maltopentaose.

TABLE II

Glucose transfer from UDP-glucose-C14 to saccharides

The standard reaction mixture, containing 0.22 μmole of UDP-glucose-C14 (12,000 c.p.m.), was incubated for 3 hours with 1 μmole of additions indicated. Results are in mμmoles.

Experiment No.	Addition	Starch formed1	Oligosaccharides formed2	Sum	UDP formed3
1	None	8.3	2.6	10.9	9.2
	Glucose	4.2	1.0	5.2	5.7
	Maltotriose	4.7	16.2	20.9	21.5
2	None	14.0	3.0	15.0	
	Salicin	10.3	0.9	11.2	
	Sucrose	12.1	2.6	14.7	
	Fructose	12.6	3.3	15.0	
	Gentiobiose	7.7	0.7	8.4	
	Cellobiose	8.2	4.6	12.8	

1 Calculated from radioactivity of starch washed with 50 % ethanol.

2 Calculated from radioactivity of the fraction soluble in 50 % ethanol and not removed by mixed-bed resin.

3 Measured with pyruvate kinase.

TABLE III

Glucose transfer from UDP-glucose-C14 to oligosaccharides

The standard reaction mixture, containing 0.53 μmole of UDP-glucose-C14 (7300 c.p.m.), was incubated for 3 hours. The starch was heated and precipitated with ethanol as described in "Experimental Procedures." The background counts were subtracted.

Additions	Starch	Oligosaccharides	Sum
	c.p.m.	*c.p.m.*	
None (t = 0)	0	48	48
None	700	64	764
Glucose	304	58	362
Maltose	610	775	1385
Maltotriose	284	930	1214
Maltotetraose	370	894	1264
Maltopentaose	304	634	938

The different saccharides of the maltose series from glucose to maltopentaose were tested with the results shown in Table III. In that experiment, the starch was solubilized and reprecipitated several times in order to free it completely from oligosaccharides. All of the added saccharides were found to decrease the incorporation of glucose in starch and with the exception of glucose they all led to the formation of oligosaccharides. It appears therefore that glucose inhibits the enzyme and that the di-and higher saccharides compete as acceptors with the starch present in the granules.

(*b*) *Labeled Oligosaccharides.* — Many experiments were carried out with the use of labeled glucose, maltose, maltotriose, and maltopentaose, with and without unlabeled UDP-glucose.

Incubation with labeled glucose, with or without added oligosaccharides, did not give rise to the incorporation of radioactivity in starch or in oligosaccharides.

Incubation of the enzyme with labeled maltose, maltotriose, or maltotetraose and UDP-glucose gave rise to a definite formation of the corresponding higher saccharides. None were formed without UDP-glucose.

A representative experiment with radioactive maltrotriose is shown in Fig. 6, and one with maltotetraose appears in Fig. 7.

Incubation with radioactive maltotriose either with or without UDP-glucose did not

lead to any incorporation of radioactivity in the starch. Therefore, oligosaccharides do not seem to be intermediates in the transfer of glucose from UDP-glucose to starch.

Successive Action of UDP-Glucose and Maltose — Since it seemed possible that the transfer from UDP-glucose to oligosaccharides might occur in two steps, that is first from UDP-glucose to starch and then from starch to oligosaccharides, the experiment shown in Table IV was carried out. In a first incubation, the starch was made with radioactive with UDP-glucose-C^{14}, ad it was then washed and incubated with maltose. It may be observed in Table IV that no radioactivity was transferred to maltose from starch (tube 1) and that the control (tube 3) showed that the enzyme was active during the second incubation.

Structure of Oligosaccharides — Inasmuch as the oligosaccharides produced by enzyme action were formed from $\alpha(1 \rightarrow 4)$ linked acceptors and not from others and because their behavior during chromatography on paper was identical with that of the $\alpha(1 \rightarrow 4)$ series, it seems reasonable to assume that they all belong to that series.

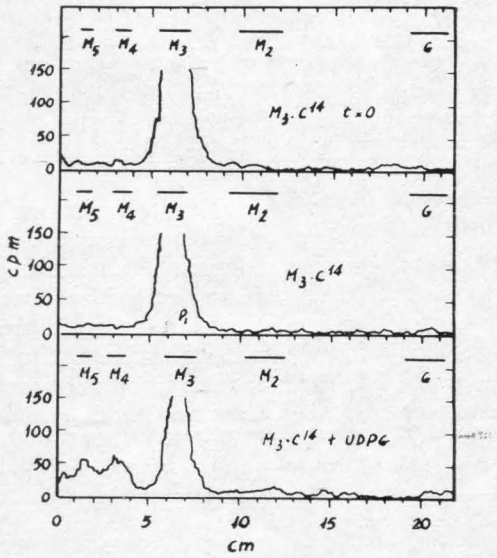

FIG. 6. — Labeled maltotriose and unlabeled UDP-glucose. Standard reaction mixture plus 0.045 μmole of maltotriose-C^{14} (5600 c.p.m.). Time of incubation was 3 hours. After paper chromatography, the radioactivity was scanned automatically. Abbreviations are as in Fig. 5.

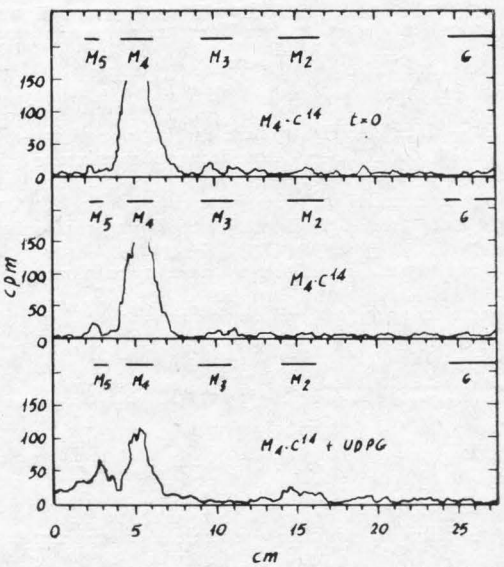

FIG. 7. Labeled maltotetraose and unlabeled UDP-glucose. Conditions as in Fig. 6, with 0.04 μmole of maltotetraose-C^{14} (5000 c.p.m.) instead of maltotriose-C^{14}. Abbreviations are as in Fig. 5.

TABLE IV

Successive action of UDP-glucose and maltose

The standard reaction mixture, containing 0.15 μmole of UDP-glucose-C^{14} (8000 c.p.m.), was used. After the first incubation (2.5 hours), the enzyme was washed twice with 0.5 ml of water and reincubated for 3 hours with buffer and the different additions. Finally, 0.5 ml of 60% ethanol was added. The soluble fraction was passed through mixed-bed resin and counted (oligosaccharide fraction). The insoluble fraction was washed 4-fold with 60% ethanol (starch fraction).

Tube	Additions during		Starch	Oligosaccharide fraction
	First incubation	Second incubation		
			c.p.m.	
1	UDP-glucose-C^{14}	Maltose	620	4
2	UDP-glucose-C^{14}		560	20
3		UDP-glucose-C^{14} + maltose	400	260
4		UDP-glucose-C^{14}	520	70

TABLE V

Borohydride reduction and bromine oxidation of oligosaccharides

The samples obtained by incubating maltotriose with UDP-glucose-^{14} (Fig. 5), or maltotriose-C^{14} with UDP-glucose (Fig. 6) with the enzyme were treated as described in "Experimental Procedure." The sample of maltotriose-C^{14} prepared as described under substrates was labeled at the reduring glucose unit.

Substance studied	Radioactivity		
	Glucose	Sorbitol	Gluconic acid
	c.p.m.	*c.p.m.*	*c.p.m.*
Borohydride reduction			
M_4* from UDP-glucose-C^{14} + M_3	112	13	
M_5 from UDP-glucose-C^{14} + M_3	70	16	
M_4 from UDP-glucose + M_3-C^{14}	17	262	
M_5 from UDP-glucose + M_3-C^{14}	14	147	
M_3-C^{14} (4000 c.p.m.)	16	417	
Bromine oxidation			
M_4 from UDP-glucose + M_3-C^{14}	12		82
M_5 from UDP-glucose + M_3-C^{14}	12		120
M_3-C^{14} (1000 c.p.m.)	10		290

* The abbreviations used are: M_3, maltotriose; M_4, maltotetraose; and M_5, maltopentaose.

Some experimets were carried out in order to find out whether the glucosyl group added from UDP-glucose became attached to the reducing or to the nonreducing end of the acceptor. The reducing sugar unit is transformed into sorbitol by borohydride reduction and hydrolysis and into gluconic acid by bromine oxidation and hydrolysis. The results of these procedures applied to oligosaccharides obtained by enzyme action (Table V) are those expected if the glucosyl group from UDP-glucose became attached to the nonreducing end of the acceptor.

DISCUSSION

There is evidence that in animal tissues, glycogen is synthesized by glucose transfer from UDP-glucose (8) and not from glucose-1-P as was believed. It has been pointed out that at least in some plant tissues (22), the ratio of inorganic phosphate to glucose-1-P is too high for the synthesis of starch via phosphorylase. Therefore, the finding of an enzyme which transfers glucose from UDP-glucose to starch may lead to a reinterpretation of present knowledge, and it may turn out that phosphorylase is mainly involved in starch breakdown.

The bean enzyme preparation which consists mainly of starch and has a very low protein content (3 μg per mg) was found to catalyze the transfer of glucose from UDP-glucose to starch and also to added maltose, maltotriose, or maltotetraose. The preparation appeared to be free from D-enzyme because it did not catalyze an exchange of radioactivity between glucose and oligosaccharides (15).

The K_m for UDP-glucose was found to have a very high value (6×10^{-2}) in relation to other enzymes; for instance, a value of 5×10^{-4} was obtained for UDP-glucose glycogen transglucosylase (9). However, it should be noted that the starch-synthesizing system consists of particles so that diffusion factors may be involved.

An interesting feature of the process is that it takes place in whole grains in which presumably the structural relation between enzyme and polysaccharide is the same as in the intact plant tissue.

SUMMARY

An enzyme has been detected in a bean starch granule preparation which catalyzes the following reaction, in which UDP is uridine diphosphate:

UDP-glucose + acceptor → UDP + α (1 → 4) glucosyl-acceptor

The acceptor may be starch or a di- or oligosaccharide of the maltose series. Other disaccharides such as sucrose, cellobiose, gentiobiose, salicin, fructose, or glucose did not act as acceptors. Glucose inhibited enzyme activity.

The conditions for maximal activity have been determined.

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Vol. 6, Nº 2, p. 85, 1961

ADENOSINE DIPHOSPHATE GLUCOSE AND STARCH SYNTHESIS

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In previous papers (Fekete *et al.*, 1960; Leloir *et al.*, 1961) an enzyme was described which catalyzes glucose transfer from uridine diphosphate glucose (UDPG) to starch or oligosaccharides. Several synthetic nucleoside diphosphate sugars have now been tested with the same enzyme preparation. They were prepared following the procedures developed by Khorana and others (Roseman, Distler, Moffatt and Khorana, 1961) with slight modifications. The most interesting result was that adenosine diphosphate glucose (ADPG) reacts about tenfold faster than UDPG. A typical experiment is shown in Fig. 1.

Addition of ADPG to radioactive UDPG led to a decrease in the incorporation of radioactivity in starch (Table I). The inverse, that is, addition of UDPG to labeled ADPG did not produce any change. Table I also shows that the difference in rate of transfer as estimated by UDP and ADP formation is also observable when incorporation of radioactivity into starch is measured.

As in the experiments with UDPG (Leloir *et al.*, 1961) the glucose transferred to starch was found to be distributed between the amylose and amylopectin components.

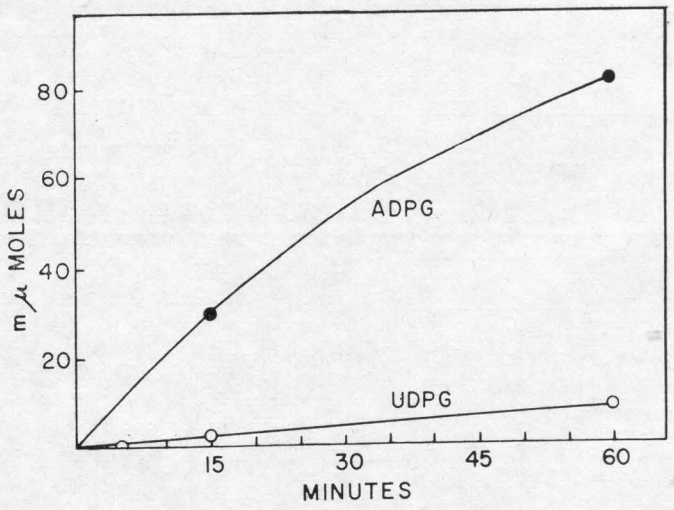

Fig. 1. — Formation of ADP or UDP. Reaction mixture (in μmoles): 0.25 of substrate, 0.1 of ethylenediaminetetraacetate, 4 of glycine buffer pH 8.4, and 1 mg of enzyme preparation. Total volume 0.02 ml. Temperature 37°. UDP (or ADP) measured with pyruvatekinase (Leloir and Goldemberg, 1960) after addition of 0.4 ml of 75 % methanol, centrifugation and evaporation of the supernatant fluid.

A transfer of glucose to added oligosaccharides takes place with ADPG as in the case of UDPG. Thus as shown in Table II, addition of maltotriose to labeled ADPG and enzyme, led to the formation of radioactive oligosaccharides and to a decrease in the incorporation into starch. Paper chromatography showed that the labeled oligosaccharide was mainly maltotetraose. The results are similar to those previously obtained with UDPG.

TABLE I

Additions		Glucose transferred to starch	
		c.p.m.	mμmoles
UDPG-C^{14} (9,500 c.p.m.)	—	242	6.4
UDPG-C^{14} (9,500 c.p.m.)	+ ADPG	58	1.5
ADPG-C^{14} (1,000 c.p.m.)	—	478	72.0
ADPG-C^{14} (1,000 c.p.m.)	+ UDPG	488	73.0

Reaction mixture as in figure 1 with 0.25 μmole of substrates (except ADPG-C^{14} of which 0.15 μmole was added. It was prepared with equimolecular amounts of adenosine 5'-phosphate morpholidate and glucose 1-phosphate-C^{14}). Total volume 0.03 ml. Incubation time 50 min. Measurements as described by Leloir *et al.* (1961).

TABLE II

Additions		c.p.m.	
		Oligosaccharides	Starch
ADPG-C^{14} (2,100 c.p.m.)		0	996
ADPG-C^{14} (2,100 c.p.m.)	+ maltotriose	410	632

Reaction mixture as in figure 1. Maltotriose 0.67 μmole. Total volume 0.03 ml. Incubation time 1 hour.

No formation of nucleoside diphosphate was detected when the enzyme was incubated with any of the following compounds: inosine diphosphate glucose (obtained by deamination of ADPG with nitrous acid), cytidine diphosphate glucose, guanosine diphosphate glucose, ADP-maltose, ADP-galactose or UDP-galactose. Furthermore the β-anomers of ADPG and UDPG gave negative results, as also did an isomer of ADPG in which the glucose is joined to ADP through position 6.

ADPG has been tested with several other systems which are known to use UDPG. The synthesis of glycogen by muscle (Goldemberg, 1961), liver, and yeast preparations was about half as fast when ADPG was substituted for UDPG. Other systems which were tested with ADPG and gave negative or nearly negative results were: the galactowaldenase system (Caputto *et al.*, 1950), and sucrose or sucrose phosphate synthesis (Cardini *et al.*, 1955; Leloir and Cardini, 1955).

From the above mentioned results it seems that ADPG and UDPG react with the same enzyme and the question arises as to whether ADPG has a role in starch synthesis "in vivo'.' There is one fact which indicates that ADPG may be a normal metabolite, and this is the presence of an ADPG-pyrophosphorylase in plant material. This enzyme which is being studied in this laboratory by Dr. J. Espada catalyzes the formation of ADPG from adenosine triphosphate and glucose 1-phosphate. It seems to be a different enzyme from UDPG-pyrophosphorylase.

ACKNOWLEDGMENTS

This investigation was supported in part by a research grant (Nº G-3442) from the National Institutes of Health, U. S. Public Health Service, by the Rockefeller Foundation and by the Consejo Nacional de Investigaciones Científicas y Técnicas.

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BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Vol. 12, Nº 3, p. 204, 1963

ISOLATION OF ADENOSINE DIPHOSPHATE D-GLUCOSE FROM CORN GRAINS

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Adenosine diphosphate D-glucose (ADPG) has been found to react about ten-fold faster than uridine diphosphate D-glucose (UDPG) in the synthesis of starch (Recondo and Leloir, 1961). Subsequently some evidence was obtained indicating that ADPG is a natural compound. Thus a specific enzyme which catalyzes ADPG formation from adenosine triphosphate and glucose 1-phosphate was isolated from wheat flour (Espada, 1962). Furthermore Kauss and Kandler (1962) observed that after administering $^{14}CO_2$ to Chlorella a radioactive compound which cochromatographed with synthetic ADPG could be detected. Recently, a phosphorylase which acts preferentially on ADPG and catalyzes its phosphorolysis to adenosine diphosphate and glucose 1-phosphate has been found in wheat germ (Dankert, Gonçalves and Recondo, 1963). The present communication reports the isolation of ADPG from an alcoholic extract of corn grains.

Five kilograms of sweet corn grains in the milky stage were disintegrated in 10 liters of ethanol (95 %) with a blendor and filtered. The extract was precipitated with mercuric acetate as described by Caputto, Leloir, Cardini and Paladini (1950). After decomposing the mercury salts with H_2S the nucleotides were adsorbed on charcoal and eluted with ethanol-ammonia-water (25:0.50:75). Aliquots were then submitted to paper chromatography in ethanol-ammonium acetate of pH 7.5 (Paladini and Leloir, 1952) (Whatman 17 paper). A band with the same mobility of a synthetic ADPG was eluted and rechromatographed in ethanol-ammonium acetate of pH 3.8 (Paladini and Leloir, 1952). After a final chromatography in the pH 7.5 solvent, a single band with the mobility of ADPG was isolated. About 8 μmoles of ADPG were obtained per kilogram of corn grain.

The ultraviolet spectrum of the compound was identical to that of adenosine in acid, neutral or alkaline medium. The substance moved like ADPG during paper electrophoresis in sodium carbonate-sodium bicarbonate buffer of pH 9.2 and sodium phosphate buffer of pH 7.5. Chromatography in ethanol-ammonia-water (Paladini and Leloir, 1952) produced adenosine monophosphate and a cyclic sugar phosphate as does synthetic ADPG.

After acid hydrolysis at pH 2 and 100º for 10 minutes, the substance gave adenosine diphosphate, some adenosine monophosphate and traces of adenine, as was shown by chromatography in the neutral ethanol-ammonium acetate solvent. Paper chromatography of the hydrolysis products in butanol-pyridine-water (Jeanes, Wise and Dimler, 1951) and paper electrophoresis in potassium tetraborate followed by treatment with the silver nitrate-sodium hydroxide reagent (Trevelyan, Procter and Harrison, 1950) revealed a large glucose spot, and smaller ones in the zones corresponding to galactose and mannose. Adenine was the only ultraviolet absorbing product produced by hydrolysis of the ADPG fraction in 3 N HCl at 100º during 1 hour, as judged by chromatography in isopropanol-HCl-water (170:41:39) (Wyatt, 1951).

The ratio adenosine-total phosphate-reducing sugars expressed as glucose was 1:1.9:1.04. Total phosphate was determined by the Fiske and Subbarow method (1925) and reducing power by the Somogyi (1945) and Nelson (1944) methods.

* Fellows of the Consejo Nacional de Investigaciones Científicas y Técnicas.

An enzymatic assay with glucose oxidase (Huggett and Nixon, 1957) after acid hydrolysis showed that 60 % to 70 % of the reducing sugar was glucose. The nucleoside diphosphate sugar was tested with the starch synthesizing enzyme from beans (Leloir, Fekete and Cardini, 1961) and the orthophosphate adenylyl transferase (Dankert, Gonçalves and Recondo, 1963) from wheat germ. The results are shown in Table I.

Apparently the substance isolated is mainly ADPG contaminated with other nucleotides of similar structure. Further studies are being carried out to clarify the nature of the contaminating nucleotides.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Drs. Carlos E. Cardini and Joaquín Espada for their help and advice and to Noel & Co. Ltd. for collaboration in the large scale step.

This investigation was supported in part by a research grant (Nº G-3442) from the National Institutes of Health, U. S. Public Health Service, by the Rockefeller Foundation and by the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

TABLE I

Enzyme	Product measured	ADPG from corn (100 mμmoles)[1]	Synthetic ADPG (100 mμmoles)[1]
		mμmoles	mμmoles
Starch synthetase	Adenosine diphosphate[2]	45	90
Orthophosphate adenylyl transferase	Adenosine diphosphate[2]	50	66
	Glucose 1-phosphate[3]	40	57
Glucose oxidase	Glucose	70	100

1 Calculated from absorbancy at 260 mμ.

2 Determined with pyruvate kinase (Cabib and Leloir, 1958).

3 Measured with phosphoglucomutase-glucose 6-phosphate dehydrogenase (Munch-Petersen, 1955).

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PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 53, N° 1, p. 86, 1965

IN VITRO SYNTHESIS OF PARTICULATE GLYCOGEN *

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High-molecular-weight glycogen was first extracted from liver by Lazarow [1] who called it particulate glycogen. Since then, it has been studied by several workers, [2,3] but it is not known whether it is composed of α-1,4 and α-1,6 linked glucose residues only or if, in addition, it has other types of linkages or materials.

The molecular weight of particulate glycogen has been estimated [2] to be $50–200 \times 10^6$ that of glycogen extracted with cold trichloroacetic acid [4] $5–70 \times 10^6$, and values of $1–3 \times 10^6$ have been assigned to alkali-extracted glycogen. [4]

When observed with the electron microscope, [5,6] liver particulate glycogen appears as clusters or rosettes of 60–200 mμ diameter composed of subparticles of 20–40 mμ diamter. The rosettes and the subparticles have been referred to as α and β particles, respectively by Drochmans. [5]

The mechanism of glycogen synthesis is fairly well known [7-9] but, to our knowledge no work has been done on the *in vitro* synthesis of particulate glycogen. It was reasoned that starting with a high donor/acceptor ratio it should be possible to attain very high molecular weights. In fact, by incubating glucose 1-phosphate, glycogen, and purified enzymes in the right proportions, it has been possible to prepare glycogen which appears to be the same as that obtained by cold water extraction of liver. By a similar procedure, Illingworth, Brown, and Cori [10] had prepared glycogen of a molecular weight: 25×10^6.

It is generally believed that the natural donor for glycogen synthesis is uridine diphosphate D-glucose, [7,8,11] but it was found more convenient to use glucose 1-phosphate and phosphorylase because they can be obtained in quantity and in pure form. Since glycogen synthetase and phosphorylase have the same acceptor specificity, [8,12] it seemed likely that the polysaccharide formed would be the same.

Materials and Methods. — Crystalline phosphorylase *b* from rabbit muscle and purified branching enzyme from rat liver were prepared as described by Fischer, Krebs, and Kent [13] and Krisman, [14] respectively. Glycogen and branching activity were measured according to Krisman. [14,15]

Particulate glycogen was extracted from the livers of sucrose-fed rats. Homogenization was carried out in 3 vol of 0.1 *M* glycine buffer, pH 10.4, followed by centrifugation as described previously. [16] The glycogen pellet was resuspended in the glycine buffer, and proteins were removed with chloroform and isoamyl alcohol as described by Sevag, Lackmann, and Smolens. [17] Alkali-extracted glycogen was prepared as described by Somogyi [18]

Electron microscopy was carried out after negative staining with phosphotungstate. The procedure was as follows: grids, covered with Formvar and coated with carbon, were immersed in a detergent solution (1% Triton X-100), washed with distilled water, and dried. A thin film of glycogen solution (about 2%) was extended over the grids with a wire loop. Excess liquid was sucked off with filter paper, and then a film of 2–4 per cent phosphotungstate was applied in a similar manner. The latter was prepared by neutralizing phosphotungstic acid with tris (hydroxymethyl) aminomethane. Observations were carried out in the electron microscope (Siemens-Elmiskop I) with a double condenser, and photographs were taken at 30,000 or 40,000 direct magnifications.

Results. — *In vitro synthesis:* The sedimentation of different glycogen samples was

studied in preliminary experiments. After centrifugation at 11,300 × *g* for 10 min, it was found that the sedimentation of particulate glycogen was 78 per cent while that of alkali-extracted glycogen was only 7 per cent.

The results of incubating glucose 1-phosphate, phosphorylase, and branching enzyme are shown in Table 1. It may be observed that in sample 1, 76 per cent of the glycogen was sedimentable. In the other samples, the proportion of sedimentable glycogen formed depended on the amount of acceptor present. Thus, sample 5 in which glycogen was present only as an impurity of some of the reagents gave the highest value (93%). In contrast, sample 7 in which more glycogen was added initially gave a value of only 10 per cent. In sample 8, in which a larger amount of alkali-extracted glycogen was added but glucose 1-phosphate was omitted, no sedimentable glycogen was formed. This shows that the proteins (phosphorylase and branching enzyme) do not produce aggregation by themselves.

The absorption maximum in the presence of iodine was at 460 mμ both for the glycogen synthesized *in vitro* and for the particulate glycogen isolated from liver. In some experiments in which phosphorylase was relatively more active than the branching enzyme, the polysaccharide gave at first a purple color with iodine, similar to that given by amylopectin. On further incubation or on addition of more branching enzyme the color became brown like that of glycogen.

Electron microscopy: When observed with the electron microscope, the particulate glycogen isolated from rat liver (Fig. 1*A*) appeared as particles of various sizes. The diameter of most of them was 80-100 mμ, and the extreme values were 20 and 180 mμ. The subparticles had a diameter of 10-24 mμ. The results were therefore similar to those described by other workers.

The glycogen prepared *in vitro* (Fig. 1*B*) with the complete system as described in Table 1, was composed of particles of slightly more uniform size, most of them of 90-100 mμ diameter with extreme values of 40 and 160 mμ. The subparticles had a diameter of 14-25 mμ. The general appearance was identical to that of particulate glycogen isolated from liver. Figure 1*C* shows, for comparison, the appearance of alkali-extracted glycogen.

Effect of dilution: As shown in Table 2, dilution of the glycogen-forming system did

TABLE 1

In vitro FORMATION OF PARTICULATE GLYCOGEN

	Sample no.	KOH-glycogen added (mg)	Final glycogen (mg)	Per cent sedimentable*	λ maximum with iodine† (mμ)
1	Complete system	0.01	3.6	76	460
2	No glucose 1-phosphate	0.01	<0.2	—	—
3	No phosphorylase *b*	0.01	<0.2	—	—
4	No branching enzyme	0.01	—	—	Blue color
5	No glycogen	—	3.15	93	460
6	No additional adenosine 5'-phosphate	0.01	0.92	55	—
7	Complete plus additional glycogen	0.33	3.5	10	460–460
8	No glucose 1-phosphate, excess glycogen	3.3	3.15	0	—

The complete system contained: 35 μmoles of glucose 1-phosphate, 0.13 μmoles of adenosine 5'-phosphate, 17 μg of phosphorylase *b*, 0.1 ml of liver branching enzyme. Total volume 0.22 ml. After 30 min. at 37°, 0.2 ml of 0.1 *M* glycine buffer of pH 10 was added. The branching enzyme was estimed according to Krisman[14]. Ten μl of the preparation gave a Δ absorbancy at 520 mμ of 0.47 per 30 min.

* The samples were analyzed for glycogen before and after centrifugation at 11,300 × *g* for 10 min. The difference was taken as sedimentable glycogen.

† Under the conditions described by Krisman.[15]

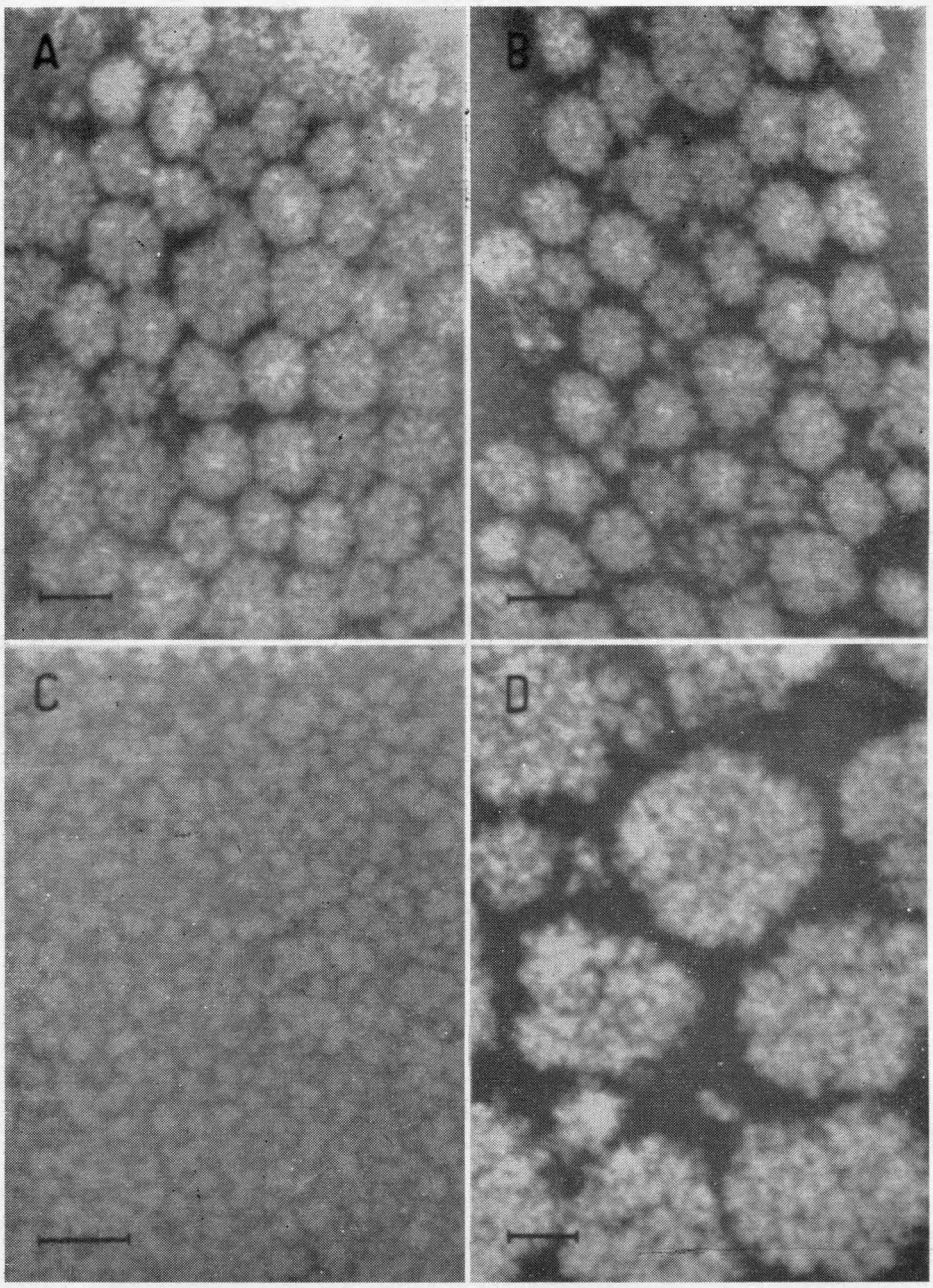

FIG. 1. — (*A*) Particulate glycogen extracted from rat liver. Negative staining with phosphotungstate, 90,000 ×. (*B*) Glycogen prepared *in vitro* as described in Table 1, 90,000 ×. (*C*) Glycogen extracted with alkali, 120,000 ×. (*D*) Overgrown particles prepared as described in text, 90.000 ×. The line in the lower left corner is 100 mμ.

TABLE 2

EFFECT OF DILUTION

Sample no.	Final volume (ml)	Incubation time (min)	Glycogen formed (mg)	Per cent sedimentable
1	0.13	45	2.1	88
2	0.27	75	2.0	90
3	0.41	120	1.7	88

Complete system as in Table 1 but with different total volumes.

not decrease the proportion of sedimentable glycogen formed.

Overgrown molecules: In order to ascertain whether there is an upper limit for the size of the particles, glycogen was prepared as described for sample 5 in Table 1. After incubation, glycogen was separated by centrifugation and treated again with enzymes and substrate. The procedure was repeated three times. This glycogen could be sedimented with a very low centrifugal force: for instance, 80 per cent of it was sedimented at 700 $\times$ *g* for 20 min, while only 6 per cent of the liver particulate glycogen sedimented under the same conditions. Observation with the electron microscope (Fig. 1*D*) revealed enormous particles of up to 380 mμ diameter. The subparticles had a diameter of 15-25 mμ.

Discussion. — The glycogen formed *in vitro* from glucose 1-phosphate appears to be identical to particulate glycogen isolated from liver, as judged by sedimentation in the centrifuge and its appearance when observed with the electron microscope. This fact permits some deductions on the nature of the link which joins the subparticles. Since purified enzymes were used for the *in vitro* synthesis, it seems very unlikely that any other type of bond in addition to the α-1,4 and α-1,6 glucosidic linkages could have been formed. Low-molecular-weight glycogen did not become particulate in the absence of glucose 1-phosphate, so that none of the proteins used in the test produced aggregation *per se*.

A possibility which has to be considered is that as the subparticles grow, they may become entangled so that larger aggregates are formed. If this were the case, it would be expected that less particulate glycogen would be formed on dilution. In fact, no change was observed on increasing the volume 3-fold (Table 2).

It has been shown that liver particulate glycogen can be obtained practically free from protein and that treatment with urea, detergents, trypsin, or chymotrypsin [2] does not cause disaggregation. According to this evidence and that presented in this paper, it appears unlikely that particulate glycogen contains any other type of bond besides the glucosidic. However, it has been pointed out that this type of glycogen is degraded by acid or alkali at a rate which seems to be greater than that of alkali-extracted glycogen. This fact might be explained as follows. Assuming that glycogen has a regular structure in which the branches divide into two at regular intervals, Pollard [19] calculated that there is a maximum limit in molecular dimensions when the diameter reaches 26-40 mμ. When this size is attained, further growth becomes impossible because no more glucose residues can be packed in a sphere of that diameter. The maximum diameter calculated by Pollard corresponds to that of the subparticles observed with the electron microscope. Growth beyond that size could occur, but irregularly. A branch could grow out of the subparticle and thus start another particle; repetition of this process would give rise to particulate glycogen. The bonds between subparticles would be α-1,4 glucosidic but they would be expected to break easily under the strain produced by the thermal agitation of the subparticles. This would explain the lability of particulate glycogen.

Summary. — Incubation of glucose 1-phosphate with crystalline phosphorylase and purified branching enzyme leads to the formation of particles which are undistinguishable from particulate glycogen as judged by their rate of sedimentation or by their appearance when observed with the electron microscope. The structure of particulate glycogen is discussed in view of the evidence presented.

The authors are grateful to Dr. H. N. Torres for the gift of crystalline phosphorylase, to Dr. B. A. Houssay for making available the electron microscope, and to Dr. C. Vasquez for advice on the negative staining procedure.

* This investigation was supported in part by a research grant (GM 03442) from the National Institutes of Health, U.S. Public Health Service, by the Rochefeller Foundation, and by the Consejo Nacional de Investigaciones Científicas y Técnicas (República Argentina).

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 121, Nº 3, p. 769, 1967

PROPERTIES OF SYNTHETIC AND NATIVE LIVER GLYCOGEN [1]

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The properties of high molecular weight glycogen extracted from rat liver and of that prepared *in vitro* with muscle phosphorylase and liver branching enzyme have been compared. The stability at different pH values was measured spectrophotometrically for liver, corn, and synthetic glycogen. The former is more labile, but the shape of the pH-stability curve is very similar for all of them. Borate, copper, and iron accelerate the decomposition of the three types of glycogen. Sonication produces breakdown but affects in the same way synthetic and liver glycogen. After shortening the outer chains with β-amylase, native liver glycogen becomes slightly more stable to acid treatment and decomposes giving smaller molecules than the untreated glycogen. Glycogen extracted from livers of toad and pigeon was similar in molecular weight distribution and acid lability to that of rat liver. Rat muscle glycogen had a molecular weight of about 8 million.

High molecular weight liver glycogen (particulate glycogen) has been studied by several methods. Estimates of the sedimentation coefficients give values which range from less than 100 up to 10,000S (1, 2). These values would correspond approximately to molecular weights of less than 10 to more than 3000 million. Observations with the electron microscope show large particles of 60-200 mμ diameter composed of 20-40 mμ subunits. These were referred to as α-and β-particles, respectively, by Drochmans (3). On the basis of the action of detergents, urea, and enzymes, Orrell *et al.* (4) reached the conclusion that the subunits are not held together by protein, hydrogen bonds, or nucleic acids.

Mordoh *et al.* (5) obtained preparations of high molecular weight from glucose 1-phosphate with purified phosphorylase and branching enzyme. Observations with the electron microscope showed that these synthetic preparations were similar to those of native liver glycogen. However, further work (1) showed that the latter is more labile to heat, alkali, and acid. The type of breakdown is also different because the molecular weight of synthetic samples decreases progressively while native liver glycogen gives preferentially molecules of 8 million daltons. Mordoh *et al.* (1) suggested that native liver glycogen might have one "labile" bond for every 50,000 glucose residues. This work has been continued, and a more detailed analysis of the breakdown of both glycogens has been carried out.

EXPERIMENTAL PROCEDURE

Synthetic glycogen was prepared as described previously (5). Native liver glycogen was obtained by the $HgCl_2$ method (1) followed by dialysis. Rat muscle glycogen was extracted by homogenizing in 8 volumes of neutralized 3 % $HgCl_2$ for 2 minutes in a blender. After two extractions the yield was about 70 % of that obtained with the usual KOH method (6). It was verified that with this treatment the molecular weight of native liver glycogen did not change. Corn glycogen was obtained as previously described (1).

The sedimentation rate of glycogen in sucrose gradients was measured according to Mordoh *et al.* (1). In some cases larger scale separations were performed in order to obtain glycogen of more homogenous molecular weight.

Sonication was carried out with a Raytheon sonicator at 10 kilocycles per second.

1 This investigation was supported in part by a research grant (GM 03442) from the National Institutes of Health. P.S. Public Health Service; by the Rockefeller Foundation; and by the Consejo Nacional de Investigaciones Científicas y Técnicas (R. Argentina).

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The analytical methods used were as follows: glycogen according to the iodine method of Krisman (7), and nonreducing end groups with periodate as described by Fales (8). The degree of β-amylolysis was determined by incubating glycogen and a commercial preparation of β-amylase in 0.1 M citrate buffer of pH 6.4. Aliquots were taken at several intervals until the reducing power reached a plateau. The product was characterized as maltose by paper chromatography in butanol-pyridine-water (6:4:3) (9). The initial amount of glycogen was measured by the phenol-sulfuric acid method (10), and the reducing power by the Somogyi (11)-Nelson (12) method. The absorbance of glycogen solutions was measured at 500 mμ.

RESULTS

Breakdown of glycogen measured by changes in absorbance. The degradation of glycogen can be easily followed with a spectrophotometer. The absorbance per unit weight is theoretically proportional to the molecular weight, and for polydisperse materials it is proportional to the weight average molecular weight (13). Therefore for the same number of bonds hydrolyzed, the percentage decrease in absorbance should be larger for heavier fractions than for lighter ones.

When native liver glycogen is heated to 100° the absorbance decreases progressively as hown in Fig. 1. The rate of change depends on the pH and on the nature of the ions. Several of the usual analytical and graphical procedures were tried in order to express the rate of the reaction, but none of them was satisfactory.

In order to find out if the course of the reaction was similar under different conditions, the curves of decrease in absorbance were plotted against time. By changing the time scale all the points could be made to fall on the same curve. The results are shown in Fig. 1. The breakdown curve with acetate of pH 5 at 100° was plotted with the abcissa

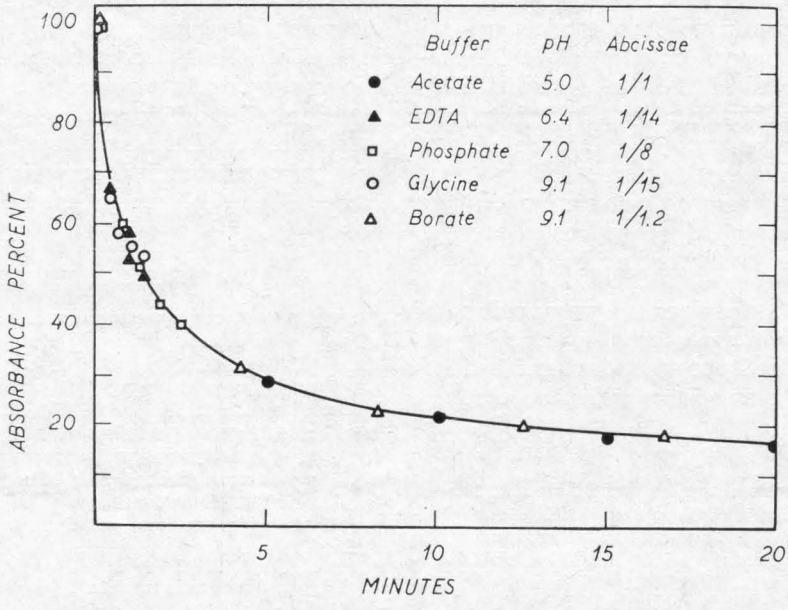

FIG. 1. — The decrease in absorbance of native liver glycogen heated to 100° in different buffers. The absorbance was measured at 500 mμ. The abcissa scale was reduced by the indicated factor. For instance the real scale for glycine would be 15 times larger The molarity of the buffers was 0.1 M.

TABLE I

Effect of Heating Glycogens under Different Conditions [a]

Addition	pH	t_{50} native	t_{83} synthetic	t_{70} corn
Sodium acetate	5	2	5.5	3
Sodium citrate	6.4	10.5	20	12
Citrate + 10^{-2} M EDTA	6.4	30	—	—
Potassium phosphate	7	11	8	8
EDTA	7	20	7.5	10
Tris-HCl	7[b]	2	—	—
Glycine-NaOH	9.1	25	30	7.5
EDTA	9.1	30	13.5	15
Diethyl barbiturate	9.1	11	11	5
2-Amino, 2-ethyl 1,3-propanediol	9.1	21	13.5	6
Sodium tetraborate	9.1	2	3.5	2
Sodium tetraborate + EDTA	9.1	2.5	3.5	—
Sodium carbonate	10	12	3.5	4
Sodium hydroxide (0.1 M)	13	4	3	2.5
Sodium hydroxide (0.05 M)	12.7	8	—	—

[a] The absorbance at 500 mμ was measured before and after heating to 100° for 5, 10, 15, and 20 minutes. The values of t_{50}, t_{83}, and t_{70} were calculated as described in text. Concentration was 0.1 M, unless otherwise indicated. The pH values are those of the solutions at 25°.

[b] pH decreases to about 5 on heating to 100°.

scale in minutes. The results obtained with other buffers are plotted with contracted abcissa scales. The results can be expressed either in t_{50}, i.e., the time at which absorbance becomes half, or as relative stability factors, i.e., the number by which the time scale has to be contracted in order to obtain superposition of the curves. For instance, in Fig. 1, if the results with acetate of pH 5 are taken as equal to one, the relative stability for glycine buffer is 15. Superposition of the curves was also obtained with synthetic and corn glycogen.

The results of heating native liver, corn, and synthetic glycogen to 100° in different buffers are shown in Table I. The decreases in absorbance were much smaller for corn and synthetic glycogen, so that results are given in t_{70} and t_{83}, respectively, instead of t_{50} used for liver glycogen. This procedure allowed comparison of the breakdown of each glycogen under different conditions but not of one type of glycogen with the others. Native liver glycogen is most stable between pH 7 and 11 and decomposes rapidly at pH 5 and 13. Sodium tetraborate accelerates the decomposition as compared with other buffers of the same pH. A rapid decomposition was also observed with Tris-HCl of pH 7; however, it was found that on heating to 100° the pH as measured with indicators decreases to about 5. The same acidification on heating occurs with buffers of other organic bases such as ethanolamine and triethylamine, but not with the other buffers. In the previous paper (1, Fig. 8) a curve is shown in which the decrease in absorbance at pH 7 is represented. At the time, it had not been noticed that such large changes in pH occurred on heating Tris buffer. The actual pH must have been near 5.

It was found that complexing agents such as glycine, EDTA, and citrate decreased the rate of decomposition, and therefore the action of some metals was tested. As shown in Table II, Cu^{++} and Fe^{3+} increased the rate of decomposition of both native liver and synthetic glycogen. Hg^{++} had the opposite effect. Citrate was used as buffer in these experiments, and the amount of free Cu^{++} can be calculated to be of the order of 10^{-3} M.

Effect of the pH on the breakdown of different glycogens. It was expected that information on the nature of the "labile" bonds of native glycogen might be obtained from the effect of pH on the breakdown. For this

TABLE II

Effect of Metals [a] *on Glycogen Stability*

Metal	t_{50}		t_{90}	
	Native	Change %	Synthetic	Change %
None	10.8	—	6.7	—
Fe^{3+}	4.0	−63	2.5	−63
Cu^{++}	5.5	−49	1.5	−78
Pb^{++}	7.5	−31	3.0	−55
Zn^{++}	8.6	−20	5.0	−25
Co^{++}	11.3	+5	5.3	−21
Hg^{++}	15.0	+39	13.3	+100

[a] The samples were heated to 100° in 0.1 M citrate plus 0.01 M of metal.

purpose samples of glycogen were heated to 100° for 5, 10, 15, and 20 minutes at different pH values. An EDTA buffer was used throughout in order to minimize the effect of metals. The relative stability was calculated as described for Fig. 1. The rates of change in absorbance for the different glycogens could not be compared directly, because in most cases the initial molecular weight was not the same and the breakdown products were different. However, the results can be expressed relative to changes at a certain pH. In this case the stability factor of each glycogen at pH 7 was taken as 100, and the respective values at different pH values were proportionally modified. The results are shown in Fig. 2. It may be observed that the curves for native liver, synthetic, and corn glycogen are similar although native liver glycogen degrades faster.

Effect of temperature on acid breakdown. The action of acid on native liver and synthetic glycogens at different temperatures was studied. When the decrease in absorbance was plotted against time it seemed that at 0° the curve became horizontal at a higher level than at 28°. But if the time scale for the 0° series is contracted, all the points fall on the same curve (Fig. 3). In order to obtain superposition of the points it was necessary to contract the 0° abcissae scale 180 and 75 times for native liver and synthetic glycogen,

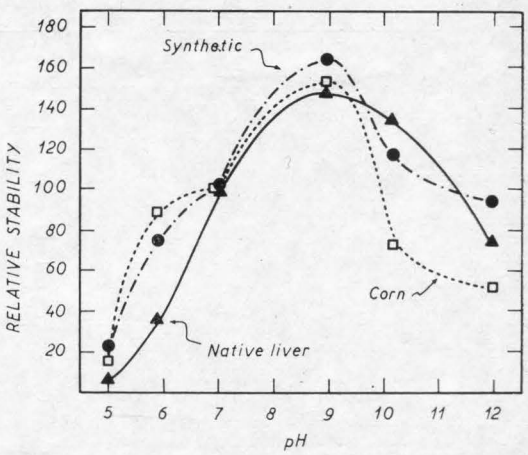

FIG. 2. — Effect of pH on the stability of synthetic, corn, and native liver glycogen. The pH values indicated correspond to the solution at 25°. They did not change more than 0.2 pH unit on heating to 100° as judged by the color of indicators. The relative stability was calculated as described in text.

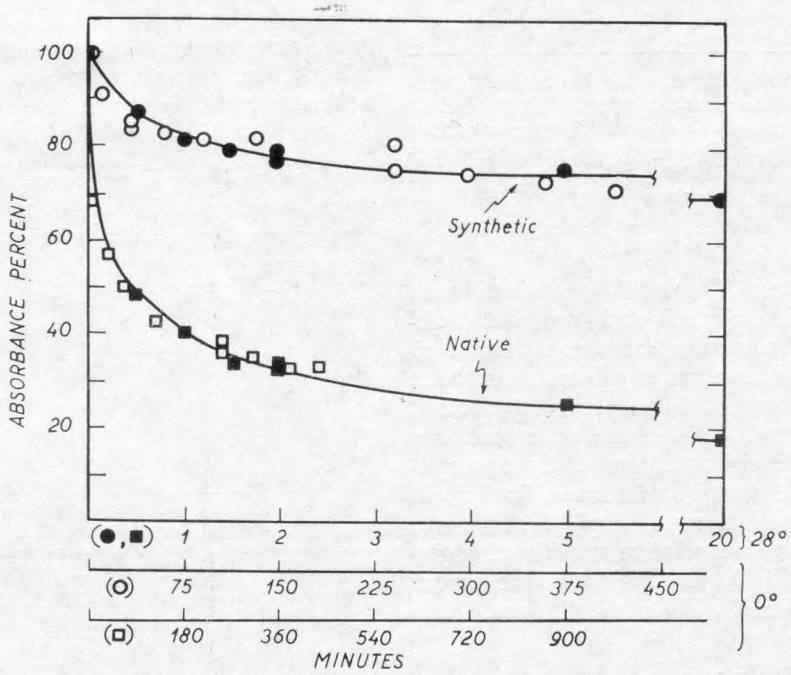

FIG. 3. — Effect of temperature on the breakdown of native liver and synthetic glycogen. The samples were maintained at the indicated temperatures in 0.1 N HCl and neutralized with a slight excess of dipotassium phosphate at different times. Three abcissa scales are drawn: one for the results at 28° and two for the values obtained at 0°.

respectively. It may be concluded that the reaction follows a similar course at different temperatures and that only the rate is changed. It was checked by gradient centrifugation that the type of breakdown of native liver glycogen at 0° in 0.1 N HCl is the same as at room temperature.

Breakdown products. It was observed previously (1) that 0.1 N HCl at 37° degrades native liver glycogen with preferential formation of molecules of 100 S which correspond to a molecular weight of about 8 million. The same type of experiments has now been carried out under other conditions. The changes in molecular weight were followed by gradient centrifugation after heating at 100° in 0.1 N NaOH, 01 M sodium tetraborate, water, or 0.1 N NaOH-8 M urea. In every case the first change was the appearance of 100S peak at the expense of the heavier fractions so that a two-peak curve was obtained. On a prolonged treatment, the heavy peak disappeared and the 100S peak was more slowly displaced to lower values.

Synthetic samples treated with 0.1 N HCl (37°), 0.1 M sodium tetraborate (100°). 0.1 N NaOH-8 M urea (100°) behaved in a different manner from native glycogen. The heavy peak was progressively displaced but there was no formation of 100S molecules.

Sonication. In the previous paper (1) it was reported that sonication affected native liver and synthetic glycogen in a different way, but the two samples used were of very different molecular weight distribution. Therefore the effect of sonication has been tested again with samples of the same molecular weight, and as shown in Fig. 4 it was found that the type of breakdown is very similar for both glycogens. The heavy fractions give rise to lighter molecules and a two-peak curve is formed. The sedimentation coefficient of the light fraction ranges between 100 and 1000S. On a prolonged treatment the heavy peak disappears and the light one becomes lighter and more homogeneous. Sonication does not lead to the preferential formation of the 100S-peak as occurs with acid or heat treatment on native liver glycogen.

Another experiment which showed that sonication breaks down native liver glycogen in a different manner than acid was carried out as follows. Two samples of native liver glycogen were degraded, one by sonication

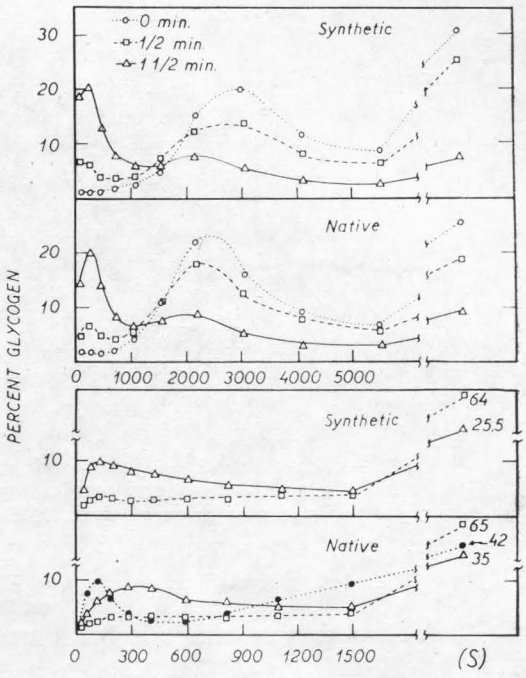

FIG. 4. — The effect of sonication. Sonication was carried out at 25° in water for the times indicated in the figure. The two upper diagrams correspond to analytical runs of 30 minutes. The time for the two lower ones was 2 hours. In the latter, undegraded natives and synthetic glycogens were omitted because nearly all of the material appeared in the pellet. In the lower diagram, the full circle (●) curve corresponds to a 100 S preparation obtained by acid treatment.

and another by acid, so as to produce samples of approximately the same molecular weight. As shown in Fig. 5, although the range of sedimentation coefficients was similar for both preparations, the acid one is less polydisperse. Both samples were then treated by 0.1 N HCl (5 minutes, 37°). Figure 5 also shows that the glycogen pretreated with acid was hardly affected, whereas that obtained by sonication gave rise to the formation of a considerable amount of a lighter component of 75S.

It therefore seems that the bonds broken down by acid are different from those affected by sonication.

End group measurement and β-amylolysis. Samples of synthetic and native liver glycogen of approximately the same molecular weight were used for measurements of the number of end groups with periodate and of the degree of β-amylolysis. As shown in Table III, the two samples did not differ signi-

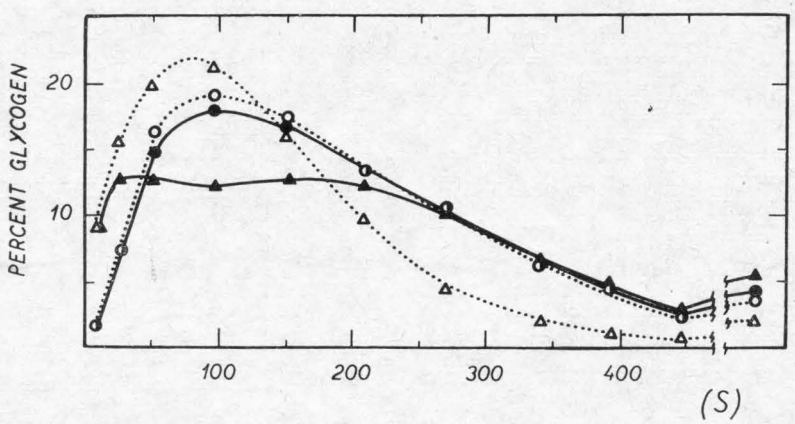

FIG. 5. — The effect of acid after sonication. Native liver glycogen was degraded with 0.1 N HCl at 37° for 10 minutes or by sonication for 12 minutes in 8 M urea at room temperature. so as to obtain a population of about 100S. Both samples were then treated with 0.1 N HCl at 37° for 5 minutes, and the rate of sedimentation in sucrose gradients was measured (15,000 rpm for 300 minutes). (▲) 100S obtained by sonic treatment ("100S sonic"); (Δ) "100S sonic" after acid treatment; (●) 100S obtained by acid treatment ("100S acid"); (o) "100S acid" after acid treatment.

ficantly. After an acid treatment which lowered the molecular weight to about the same extent, the degree of β-amylolysis increased equally in both samples. Presumably this increase was due to the fact that after acid treatment some long internal or "buried" external branches became available to enzyme action.

Other samples of synthetic glycogen had longer outer branches and some of them gave β-amylolysis values of 50-55 % before the acid treatment.

Effect of acid on β-limit dextrins. Treatment of native liver glycogen with β-amylase led to the removal of about 40 % of the glycogen residues from the exterior branches. The β-limit dextrin obtained was found to be degraded more slowly by 0.1 N acid than native samples. Figure 6 shows the course of the breakdown of the β-limit dextrin compared with a sample of native liver and synthetic glycogen of the same molecular weight. The initial distribution of molecular weights appears in Fig. 6A. After treatment with 0.1 N HCl for 1 1/2 minute, the native sample gave rise to the formation of much more of the lighter component than the β-dextrin (Fig. 6B). After 10 minutes (Fig. 6C) the native sample was all converted to a light fraction, but about 75 % of the β-dextrin remained as heavy component. The light component formed from the β-dextrin had a sedimentation coefficient of about 70, and that formed from native glycogen was, as usual, the 100S component (Fig. 6D).

TABLE III

Number of End Groups and Degree OF β-AMYLOLYSIS

Glycogen	End groupe [a] (%)	β-Amylolysis [a] (%)	β-Amylolysis after acid treatment [b] (%)
Native liver	6.90	40	53
Synthetic	6.75	42	57

a Measured as described under *Experimental Procedure.*

b The acid treatment of native and synthetic glycogen was carried out in 0.1 N HCl at 37° and 100°, respectively. The time of treatment was sufficient to decrease the initial molecular weight of about 250 million to about 8 million.

Synthetic glycogen also decreased in molecular weight but did not give rise to a lighter component.

Glycogen from other sources. Glycogens from several other sources than rat liver were prepared by the $HgCl_2$ method. Toad and pigeon liver glycogens were observed to be very polydisperse and to give sedimentation curves similar to those from rat liver. On treatment with 0.1 N HCl at room temperature they were found to be degraded rapidly to 8 million dalton molecules.

The glycogen from rat muscle was quite different since it was nearly monodisperse and had sedimentation coefficients of 90, 100, and 110S in different samples. The sedimentation pattern of one of them is shown in Fig. 7. A preparation of human muscle glycogen was examined by Bueding *et al.* (14). The sedimentation coefficient was about 130S, and observations with the electron microscope showed only small molecules.

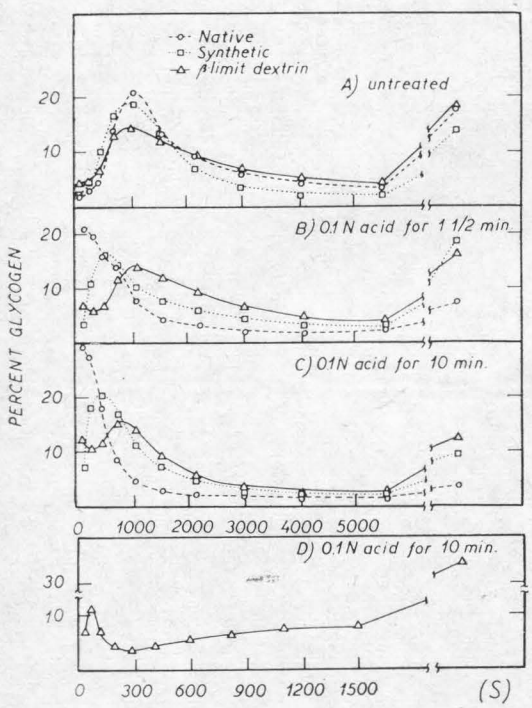

FIG. 6. — The breakdown of β-amylase limit dextrin of native liver glycogen. The three samples shown in *A* had been prefractionated so as to obtain similar molecular weigths. Acid treatment (0.1 N HCl) was carried out at 37° for the indicated times. The analytical gradients were run at 15,000 rpm for 30 minutes except for *D*, which was run 2 hours.

Electron microscopy. It was previously reported that the aspect of native liver and synthetic glycogens was similar when they were stained with phosphotungstate and observed under the electron microscope (5). However, the pictures obtained by this method are not the same for different grids or for different zones of the same grid. Reissig and Bueding (personal communication) circumvented this difficulty by spraying the samples and observing them separately and in mixture. According to them the synthetic glycogen may offer very different aspects: some samples have no subunits at all, whereas others show subunits that are less defined than in native glycogen.

In order to recognize with certainty which were the molecules of synthetic glycogen and which were those of native liver glycogen, some experiments were carried out on mixtures of light native and heavy synthetic glycogen, on the inverse mixture, and on the separate components. The results are shown in Fig. 8. The preparation of synthetic glycogen appears to have subparticles, but they are less clearly defined than in native glycogen; and the molecules have a more compact aspect.

Another point which must be considered in these observations is that synthetic samples may be more or less ramified acording to the preparation. Less ramified samples, that is, those prepared with a relative deficiency in branching enzyme and which give more purple color with iodine, give a more fluffy appearance in the electron microscope (Drochmans, personal communication).

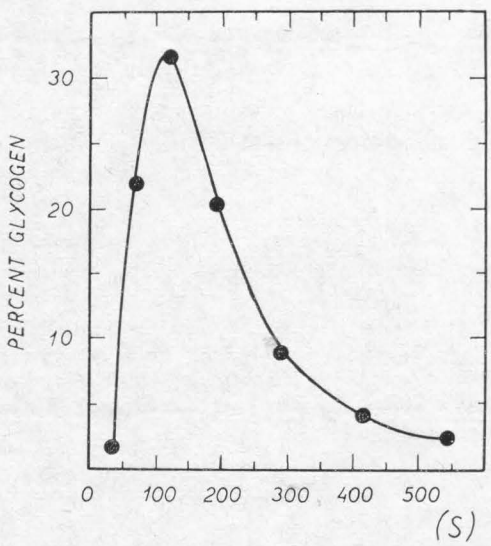

FIG. 7. — Sedimentation of rat muscle glycogen. Extraction was carried out as indicated in text, and gradient centrifugations at 15,000 rpm for 120 minutes.

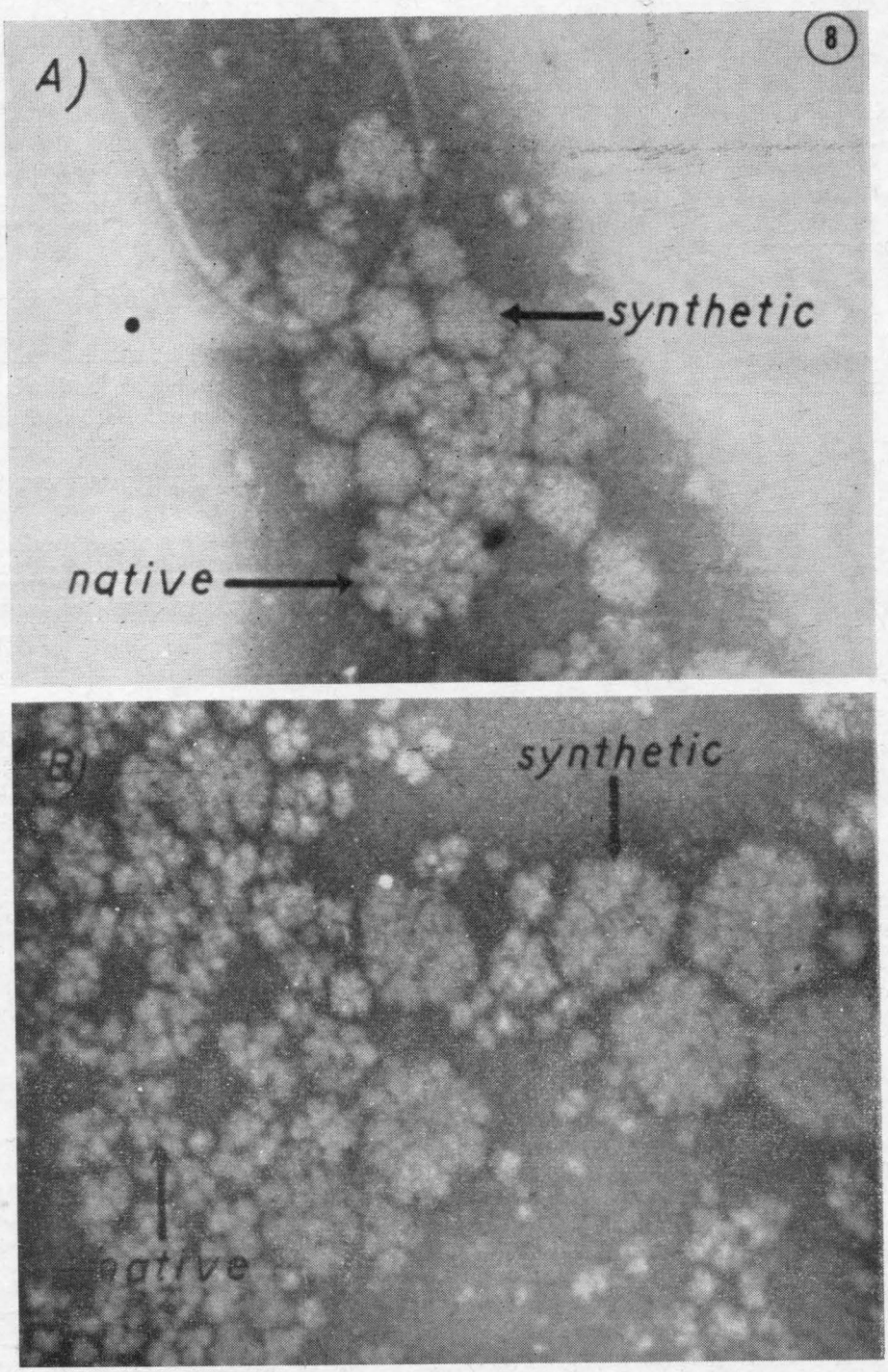

FIG. 8. — Electron microscope pictures of native liver and synthetic glycogen. Part A corresponds to a mixture of high molecular weight native glycogen and low molecular weight synthetic glycogen, and Part B to the inverse preparation. The observations were carried out as previously described (5). ×90,000.

DISCUSSION

A previous study (1) of the breakdown of native liver glycogen led to the conclusion that a small number of the bonds is more labile than the rest and than those of synthetic glycogen. The breakdown yields molecules of 8 million daltons so that the number of such bonds would be about one every 50,000 glucosidic residues. The lability of these linkages appears to be enhanced by a weight factor, that is, by the mechanical pull exerted on them. The fact that removal of the external branches by β-amylase reduces lability is in agreement with this idea. However, there must be some other cause of lability besides the weight factor. It seems that the β-limit dextrin has some bonds which, although they are not as labile as those of native glycogen, are cleaved preferentially and yield molecules of about 4.5 million daltons. Furthermore, synthetic glycogen can have molecular weights as high as the native, but is not as labile.

Several possibilities can be considered. One is that in native liver glycogen, the 8 million subparticles are joined by relatively long interconnecting chains and produce something similar to a bunch of grapes. The breakdown would then occur preferentially at the interconnecting chains. Observations with the electron microscope favor this possibility since native glycogen appears as a less compact structure with better defined subparticles. However, an observation difficult to reconcile with the grape structure is the effect of sonication. The latter would be expected to produce breakdown to the 8 million subparticles, but this is not the case. In fact the effect of sonication is nearly the same for native and synthetic glycogen.

Another explanation of the lability would be that some parts of the molecule contain residues different from α-1,4 − α-1,6 glucosyl It is known that under certain conditions, galactose (15) or glucosamine (16) residues can be introduced into the glycogen molecules. If residues such as fructofuranosyl were present they would confer acid lability to the glycogen molecule. Still another possibility is that some glucosyl residues are distributed in space so as to reduce stability by a different conformation or by decreased hydrogen bonding. The latter, however, do not seem to play an important role because the type of breakdown of native and synthetic glycogen under conditions in which no hydrogen bonds are present (100°, 8 M urea, pH 13) is respectively the same as at room temperature at pH 1, where this type of bonds should be present.

The fact that the pH-stability curve is so similar for native, synthetic, and corn glycogen indicates that a very similar type of bond is broken down in all of them. The same is true for the effect of tetraborate, copper, and iron, which accelerate decomposition of both native liver and synthetic glycogen.

If the labile bonds are-glycosidic it should be possible to label the corresponding residues by reduction with tritium-labeled sodium borohydride. Many attempts carried out in this direction failed because a considerable amount of tritium became fixed, apparently in an unspecific manner. This difficulty has been found also by De Wulf and Hers (personal communication).

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 132, Nº 1, p. 111, 1969

IN VITRO SYNTHESIS OF PARTICULATE GLYCOGEN FROM URIDINE DIPHOSPHATE GLUCOSE [1]

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High molecular weight glycogen has been prepared *in vitro* with liver glycogen synthetase (uridine diphosphate glucose: α-1,4glucan α-4-glucosyltransferase, EC 2.4.1.11) and branching enzyme (α-1,4-glucan: α-1,4 glucan 6-glycosyltransferase, EC 2.4.1.18) and uridine diphosphate glucose as glucose donor. The product obtained did not differ significantly from the native glycogen as judged by iodine spectrum, sedimentation coefficient in sucrose gradients, and by the effect of treatment with acid or alkali. Glycogen obtained from uridine diphosphate glucose differed from that prepared with glucose 1-phosphate as glucosyl donor.

The molecular weight of liver glycogen extracted in the cold and at neutral pH is very high (1–5). Values of 10–1000 million daltons have been reported (4, 5). Samples of the same molecular weight have been obtained *in vitro* by incubating glc-1-P[3], crystalline phosphorylase (α-1,4-glucan: orthophosphate glucosyltransferase, EC 2.4.1.1.) and purified liver branching enzyme (α-1,4 glucan 6-glycosyltransferase, EC 2.4.1.18) (6) but further work showed that this glycogen is different from that isolated from liver (5, 7). Glycogen prepared with phosphorylase (glc-1-P-glycogen[4]) was found to be more stable to heat, acid and alkali. The degradation of native glycogen under such conditions gave rise preferentially to molecules of about 8 million daltons whereas glc-1-P-glycogen was degraded with a progressive decrease in molecular weight.

Observations with the electron microscope showed that native glycogen appeared to have more loosely joined subparticles, giving a more open structure (7).

This paper reports the preparation and properties of glycogen obtained using UDPG and glycogen synthetase (UDPG: α-1,4-glucan α-4-glucosyltransferase, EC 2.4.1.11). The samples obtained in this manner were very similar to those of native liver glycogen as judged by the rate of sedimentation and by the effect of acid or alkali.

MATERIALS AND METHODS

Materials. The synthesis of glc-1-P and UDPG was carried out according to MacDonald (8) and Moffat (9) respectively. Yeast UDPG was a gift of C. F. Boehringed & Soehne (Germany) or was purchased from Sigma Chemical Co. (USA). Radioactive UDPG was obtained from the Radiochemical Centre (England); bovine pancreatic ribonuclease 5 × crystalline type I-A was purchased from Sigma Chemical Co. (USA) and was dissolved (10 mg per ml) in glycylglycine buffer of pH 7.75. Native liver glycogen was obtained by extraction with phenol as described by Laskov and Margoliash (10), with some modifications. The preparation of glycogen from glc-1-P was carried out as described by Mordoh *et al.* (6).

Analytical. Molecular weight determinations were carried out by centrifugation in sucrose gradients as previously described (5). The calibration data were the same. Radioactivity was measured using Bray (11) solution and a scintillation counter. Glycogen was measured according to Krisman (12). Glycogen synthetase was assayed as described by Rothman and Cabib (13) with some modifications.

Enzymes. Rat-liver branching enzyme was prepared as described by Krisman (14) but the livers were thoroughly perfused with 250 mM sucrose, 5 mM

[1] This investigation was supported in part by a research grant (No. GM 03442) from the National Institutes of Health, U. S. Public Health Service, by The Rockefeller Foundation and by The Consejo Nacional de Investigaciones Científicas y Técnicas (R. Argentina).

[2] Career investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas.

[3] The following abbreviations are used: glc-1-P, d-glucose 1-phosphate; UDPG, uridine diphosphate glucose.

[4] For simplicity glycogens synthesized *in vitro* from glc-1-P and phosphorylase or from UDPG and glycogen synthetase will be referred to as glc-1-P-glycogen and UDPG-glycogen, respertivel.

EDTA. Crystalline phosphorylase *b* from rabbit muscle was obtained according to Fischer *et al.* (15).

Liver glycogen synthetase. This enzyme was prepared as follows: Rats weighing about 300 g were fasted for 48 hr. In order to decrease glycogen content as much as possible, insulin, which is known to produce glycogenolysis in liver, was injected intraperitoneally (20 units) and the animals were killed 1 hr later. The excised livers were perfused with a solution containing 250 mM sucrose, 10 mM mercaptoethanol, 5 mM EDTA, and then homogenized in 5 vol of a similar solution but containing 880 mM sucrose. The homogenate was centrifuged at 96,000*g* for 3 hr. The precipitate was discarded and the supernatant fluid was centrifuged at 150,000*g* for 3 hr. The small pellet was resuspended (three livers in about 1 ml) in the solution used for homogenization. The resulting preparation was found to be stable for weeks when kept at −20° and could be frozen and thawed repeatedly without loss of glycogen synthetase activity. It had some branching activity and only traces of α-amylase. No glycogen was detectable in the preparations as judged by colorimetric tests but acceptor activity could be detected by carrying out measurements of glycogen synthetase activity without added glycogen. The molecular weight of this endogenous acceptor must be less than 5 million daltons since glycogen of that molecular weight is 90 % sedimentable when centrifuged at 96,000*g* for 3 hr in 880 mM sucrose.

Glycogen synthesis from UDPG. A typical incubation mixture contained 0.11 M glycylglycine buffer of pH 7.75, 44 mM mercaptoethanol, 5.5 mM glucose 6-phosphate, 22 mM EDTA (pH 7.0), 0.02 mg of KOH-glycogen, 0.1 M UDPG, 10 µl of ribonuclease solution, 150 µl of glycogen synthetase and 150 µl of the branching enzyme, in a total volume of 450 µl. The presence of chloride ions was avoided in order to keep α-amylase activity as low as possible.

After 5 hr at 37°, water was added to make 5 ml and the mixture was centrifuged at 2,000 rpm for 5 min. The supernatant fluid was shaken with 2 ml of water-saturated phenol and centrifuged as before; the phenol was re-extracted with 5 ml of water. The two aqueous layers were mixed and 2 vol of ethanol were added. The precipitate was dissolved in water and dialyzed overnight against water in the cold room. The supernatant fluid obtained after centrifugation (2,000 rpm, 10 min) was precipitated with ethanol. A drop of saturated ammonium acetate was added in order to obtain good precipitation.

RESULTS

Properties of glycogen prepared from UDPG. Previous attempts to prepare high molecular weight glycogen with UDPG and glycogen synthetase failed because the enzyme preparations were not active enough or contained amylase. Under the conditions given in Methods glycogen is formed with a yield which often reached 50 % of the theoretical based on UDPG added. The glycogen formed was difficult to free from nucleic acids and, to facilitate purification, ribonuclease was added to the reaction mixture.

The iodine spectrum of the product formed was almost the same as that of native glycogen and clearly different from that of amylopectin (Fig. 1). Treatment with salivary α-amylase gave rise, as expected, to the formation of maltotriose and maltose and under the action of β-amylase, maltose was formed.

The molecular weight distribution of a sample is shown in Fig. 2. Most of the substance is of very high molecular weight. The curves varied in different experiments (see also Fig. 4A) but did not differ much from those of native liver glycogen.

A comparison of native, UDPG-glycogen and glc-1-P-glycogen was carried out by measuring the decrease in absorbance in 0.1 N acid at room temperature or in 0.1 N alkali at 100°. Previous work (5, 7) had shown that under these conditions glc-1-P-glycogen can be clearly distinguished from native samples, because the latter is more labile. As shown in Fig. 3, UDPG-glycogen and native glycogen were found to behave exactly the same, whereas the turbidity of glc-1-P-glycogen decreased more slowly.

Another way of following the degradation of glycogen is by gradient centrifugation. Previous work (5, 7) had shown that acid or alkali treatment of native glycogen gives rise preferentially to molecules of about 8 million daltons, whereas glc-P-glycogen showed a progressive decrease in molecular weight.

The action of acid on native glycogen and UDPG-glycogen is shown in Fig. 4. Before

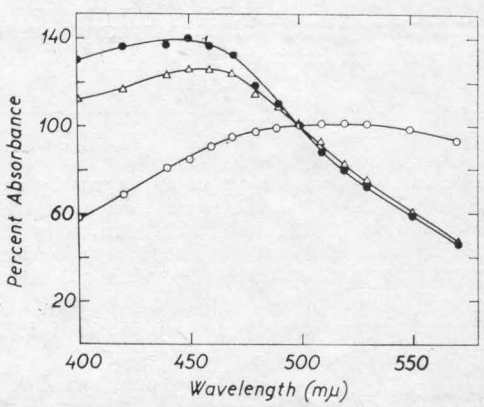

FIG. 1. — Iodine spectra of liver glycogen, amylopectin and UDPG-glycogen. Conditions as described by Krisman (12). Absorbances at 500 mµ are taken as 100 %. Full circles: native liver glycogen; empty circles: amylopectin; triangles: UDPG-glycogen.

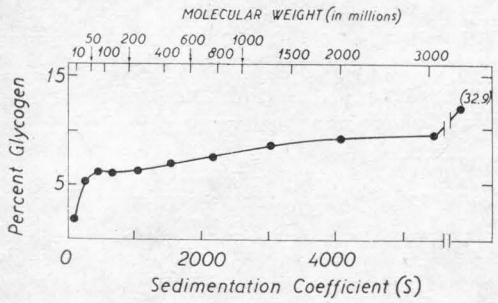

FIG. 2. — Molecular weight distribution of glycogen prepared with UDPG as glucosyl donor as described in Methods. The number in parentheses represents the percentage glycogen in the pellet. This corresponds to glycogen of sedimentation coefficient higher than 5500S plus that which sediments along the walls of the tube.

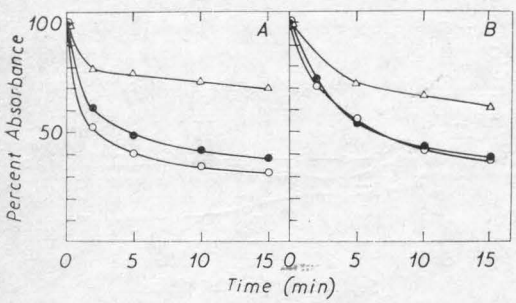

FIG. 3. — The action of acid and alkali on native glycogen, UDPG-glycogen and glc 1-P-glycogen. Absorbance was measured at 500 mμ. A: 0.1 N HCL at 25°; B: 0.1 N NaOH at 100°. Full circles: native glycogen; empty circles: UDPG-glycogen; triangles: glc-1-P glycogen.

treatment both samples gave similar curves (Fig. 4A). After 30 sec in 0.1 N acid at 37° a peak appeared at about 260S (Fig. 4B) and after 5 min nearly all the population had become light (Fig. 4C). A fine comparison of the light peak was obtained by centrifuging for a longer time. In this way slight differences were detectable between the two samples. It can be observed in Fig. 4D that at 30 sec the light peak of UDPG-glycogen is more polydisperse than that of the native. After a 5- or 10-min treatment (see Figs. 4E and F) the peak values were 150S and 100S for UDPG-glycogen and native glycogen, respectively. These values correspond to 13 and 8 million daltons. The results of alkaline treatment are shown in Fig. 5 A to D. Both native glycogen and UDPG-glycogen were appreciably degraded after 1 min and became progressively lighter. No appreciable difference between the two samples was observable. However, after 5 min in 0.1 N alkali at 100° the native sample showed a small peak at about 110S which was absent in the sample synthesized *in vitro*. The result of a more drastic alkaline treatment (33 % KOH at 100° for 20 min) is shown in Fig. 6. Both samples gave rise to the formation of a glycogen of approximately the same molecular weight (8 million daltons).

A comparison of the decomposition of UDPG- and glc-1-P-glycogens produced by heating at a pH of about 5 is shown in Fig. 7. It can be seen that the initial curves were very similar but after heating a peak at about 180S was formed from UDPG-glycogen whereas glc-1-P-glycogen was only slightly degraded.

Treatment with 8 M urea, which is currently used for breaking hydrogen bonds, produced no appreciable changes on the UDPG-glycogen samples. The treatment was carried out at 37° for 10 min. It had been observed before (5) that native glycogen is slightly degraded after 7 days in 8 M urea.

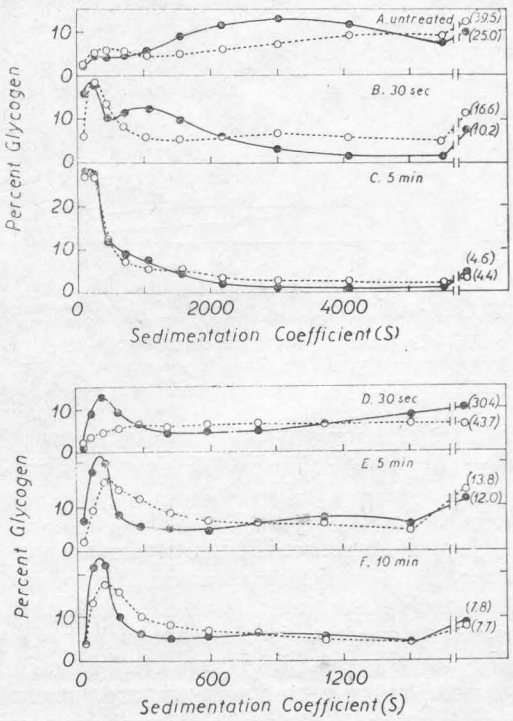

FIG. 4. — The action of acid on native glycogen and UDPG-glycogen. Full line: native glycogen; dotted line: UDPG-glycogen. A: before treatment; B-F: after indicated times in 0.1 N HCl at 37°. The centrifugation time was: A-C: 33 min; D-F: 121 min.

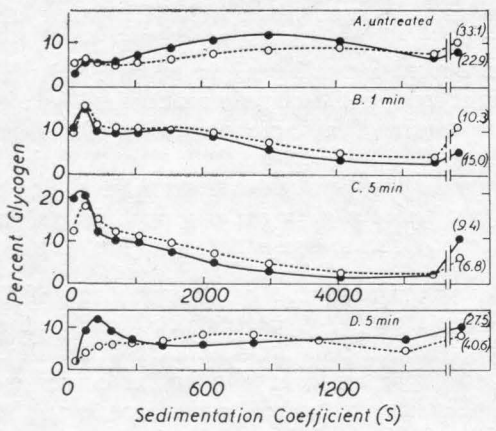

FIG. 5. — The action of alkali on native glycogen and UDPG-glycogen. Full line: native glycogen; dotted line: UDPG-glycogen. A: before treatment; B-D: after indicated times in 0.1 N NaOH at 100°. The centrifugation time was: A-C: 33 min; D: 121 min.

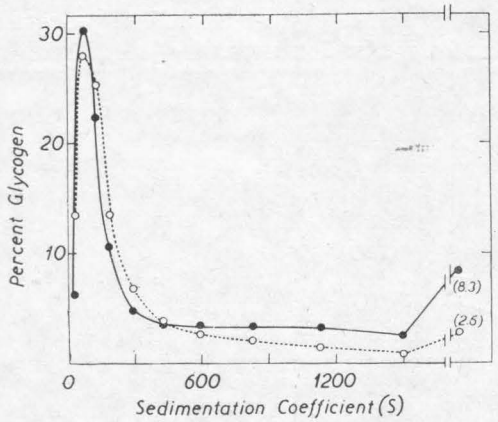

FIG. 6. — The action of alkali on native glycogen and UDPG-glycogen. Full line: native glycogen; dotted line: UDPG-glycogen. The original samples were the same as those shown in Fig. 5A. They were treated with 33 % KOH for 20 min at 100°. The analytical run in sucrose gradient was for 121 min.

Effect of dilution. If high molecular weight glycogen is formed by entanglement of molecules during synthesis it might be reasoned that if the reaction is carried out in a dilute solution, the resulting glycogen should be lighter. The result of such an experiment is shown in Fig. 8. One of the incubation mixtures was diluted six-fold with water and incubated a longer time so as to reach the same final yield of glycogen. The diluted sample gave high molecular weight glycogen whereas the control did not become as heavy in the time allowed for the synthesis to take place. The results were, therefore, the opposite from those expected from the entanglement hypothesis. No explanation for the result has been yet found.

Glucose 1-phosphate as donor. The products obtained from glc-1-P and UDPG under the same conditions were studied. One sample was synthesized with UDPG as donor and another with exactly the same incubation mixture except that UDPG was replaced by glc-1-P plus phosphorylase and adenosine 5'-

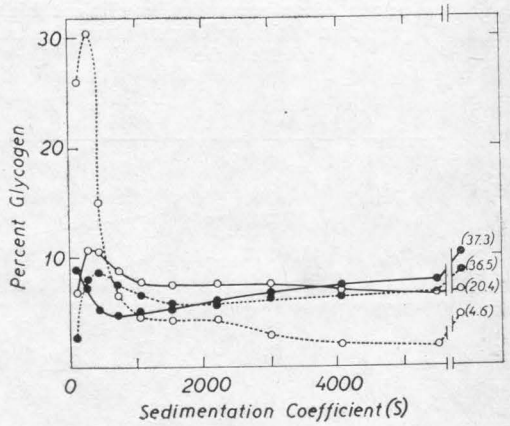

FIG. 7. — Lability of UDPG-and glc-1-P-glycogens. The samples were heated at 100° for 15 min in 0.1 M Tris-HCl buffer of pH 7.2 (the pH at 100° is about 5). Full circles: before treatment; empty circles: after heating. Full line: glc-1-P-glycogen; dotted line: UDPG-glycogen.

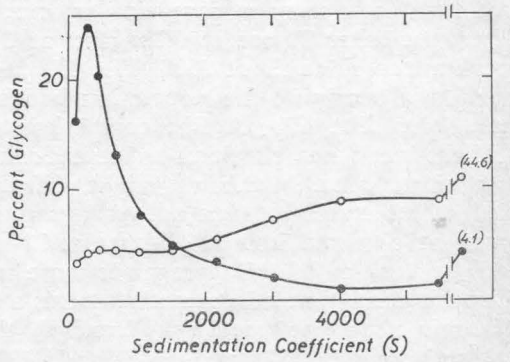

FIG. 8. — Glycogen synthesis in a diluted reaction mixture. Full circles: glycogen formed from UDPG as described in text. Empty circles: glycogen formed with the same mixture but diluted six-fold with water. The incubation times were 30 and 96 min, respectively. Radioactive UDPG was used and glycogen content was estimated by its radioactivity. Total synthesis was 15 % higher in he undiluted sample.

monophosphate. Although the extent of synthesis was the same, the molecular weight distribution was completely different, the glc-1-P-glycogen being lighter (Fig. 9). It was checked photometrically and by gradient centrifugation that the effect of acid on this sample was the same as on glc-1-P-glycogen prepared as previously described (5, 6). It should be pointed out that the glc-1-P-glycogen can reach higher sizes if KOH-glycogen is omitted in the incubation mixture.

It can be predicted that the final molecular weight should be inversely proportional to the amount of acceptor molecules present when the reaction is started. Therefore one explanation of the fact that the glycogen formed with glc-1-P is lighter than that obtained from UDPG, would be that some reagents (glc-1-P, phosphorylase or adenosine 5'-monophosphate) contained acceptor molecules. This possibility was excluded by control experiments in which each of these substances were found to produce no changes in the molecular weight distribution when added to incubation mixture in which UDPG was the glucose donor.

Variations in the incubation mixture. The omission of glucose 6-phosphate in the incubation mixture did not change appreciably the molecular weight distribution. However since the glycogen synthetase is only about 80 % active without glucose 6-phosphate the rate of synthesis was slightly slower. Ribonuclease was included in the incubation mixture because difficulties were experienced in the purification of glycogen and the main contaminant appeared to be nucleic acids. The distribution curve of glycogen obtained with or without ribonuclease was the same.

In some experiments synthetic UDPG was used as donor instead of UDPG isolated from yeast. The glycogen obtained was the same with the two samples as judged by molecular weight distribution and acid or alkaline hydrolysis. The reason for carrying out these tests was that UDPG isolated from natural sources may contain other nucleotide sugars and that the introduction of a residue different from glucose might be important in giving glycogen its lability.

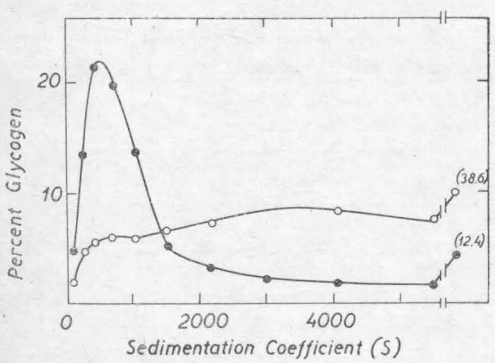

FIG. 9. — Molecular weight distributions of glycogen formed from glc-1-P and UDPG. Full circles: glc-1-P as glucose donor; empty circles: UDPG as glucose donor. The reaction mixtures were as described in Methods for UDPG but one sample contained synthetic glc-1-P (270 mM), adenosine 5'-monophosphate (4.4 mM) and crystalline phosphorylase *b* instead of UDPG. The amount of glycogen formed was practically the same in both cases.

DISCUSSION

A comparison of the glycogen synthesized *in vitro* from UDPG with that extracted from liver has shown that both exhibit similar molecular weight distribution. The rate and type of breakdown in acid or alkaline solutions is also the same with only minor differences.

The glycogen obtained from UDPG is clearly different from that prepared with glc-1-P. The latter is more stable and its molecular weight is lower when both are synthesized under conditions in which the amount of initial acceptor and of transfer are equal. These facts raise two problems. One is the explanation of the lability which has been discussed in a previous paper (7), and the other is the difference in molecular weight of the glycogens.

It was suggested previously that the lability of native glycogen might be due to the presence of residues different from α-1,4 and α-1,6-glucosyl, or to some particular distribution of the monomer units.

It does not seem likely that glycogen prepared *in vitro* from pure UDPG contains any other residue but glucosyl. However since the enzyme preparation is not pure it cannot be excluded that some unusual kind of linkage could be formed. With reference to the second problem, that is that for the same amount of acceptor and of synthesis, the glycogen obtained from UDPG is much heavier and polydisperse than that prepared with glc-1-P, no clear explanation is as yet available. The difference in the product obtained with the two enzymes may be connected to their different specificity towards the acceptor.

The transfer of labelled glucose from UDPG into a phosphorylase limit dextrin was studied by Brown *et al.* (16). The radioactive product was debranched with a pullulanase-type enzyme and it was found that only 2 % radioactivity was released from the enlarged limit dextrin (9). Therefore, transfer from UDPG occurs on the main chains only. On the other hand Brown *et al.* (17) studied the action of oligo-glucan-transferase-amylo-1,6-glucosidase on phosphorylase limit dextrins enlarged with glc-1-P and concluded that phosphorylase adds randomly to all the non-reducing groups. They also observed that the limit dextrin enlarged with UDPG gave more color with iodine than that grown from glc-1-P. That is as if transfer from UDPG led to the formation of longer branches.

Further evidence showing that glycogen synthetase adds glucose only to some of the exterior chains of glycogen is provided by the work of Kindt and Conrad (18). They found that with an enzyme from *Aerobacter aerogenes* and adenosine diphosphate glucose as donor, less than half of the non-reducing ends became glucosylated.

The results of Biely *et al.* (19) can be interpreted similarly. They used UDP-2-deoxyglucose as donor, excess glycogen as acceptor and yeast synthetase. The polysaccharide was then treated with β-amylase. The products expected from a multichain transfer would be maltose and the analog, containing glucose and 2-deoxyglucose. However, the latter product could not be detected and instead maltose and 2,2'-dideoxymaltose were found, as if repetitive transfer occurred on only some of the outer branches.

If glycogen synthetase adds glucose only to the main chains, the result would be a comb-like structure similar to that proposed by Staudinger and Husemann (20) instead of the classical Meyer and Fuld's branched tree (21). The former structure might occur in certain regions of the glycogen molecule so as to produce long chains joining subparticles. These chains would have an increased lability due to the mechanical pull exerted by the subparticles. The difference in molecular weight of the glycogens synthesized from glc-1-P and UDPG seems to be due to a process of aggregation which occurs in the latter case. This problem is being studied.

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PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 66, Nº 1, p. 153, 1970

DOLICHOL MONOPHOSPHATE GLUCOSE: AN INTERMEDIATE IN GLUCOSE TRANSFER IN LIVER *

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Abstract. The microsomal fraction of liver has been found to catalyze glucose transfer from UDPG to a lipid acceptor which appears to be identical to the compound obtained by chemical phosphorylation of dolichol. The substance formed (dolichol monophosphate glucose) is acid labile and yields 1,6-anhydroglucosan by alkaline treatment. It can be used as substrate by the enzyme system yielding a glucoprotein which is subsequently hidrolyzed to glucose.

One of the most important developments in the field of saccharide biosynthesis has been the discovery of lipid intermediates in sugar transfer reactions. The studies of Wright *et al.*[1] on O-antigen and of Higashi *et al.*[2] on peptidoglucan synthesis in bacteria showed that polyprenol pyrophosphate sugars are formed by transfer from nucleotide sugars and subsequently act as donors for polysaccharide formation. As shown by Scher *et al.*,[3] similar events occur in *M. lysodeikticus* where mannose is first transferred from GDP-mannose to undecaprenol monophosphate and then to mannan. In animal tissues an enzyme has been described which catalyzes mannose transfer from GDP-mannose to a lipid.[4]

In the course of work with UDPG it has now been found that liver contains enzymes which catalyze the following reactions:

Since the rate of formation of glucosylated acceptor lipid by reaction (1) is proportional to the acceptor lipid added, the latter could be estimated and purified. The preparations obtained could be used as substrates for reaction (2). This direct transfer from the lipid intermediate has only been detected in few cases. Evidence is presented indicating that the acceptor lipid is dolichol monophosphate. Dolichol[5] is a polyprenol present in animal tissues which has 16 to 21 isoprene units, the first being saturated.

Materials and Methods. Reagents: UDPG labeled in the glucose with ^{14}C was prepared according to the method of Wright and Robbins[6] with slight modifications. Dolichol was prepared from pig liver. The extraction and alumina chromatography was performed as described by Burgos *et al.*[5] The fractions were analyzed by thin-layer chromatography with chloroform as solvent. Those which contained a substance having an R_f of about 0.45 and giving a green color with the anisaldehyde reagent were pooled and purification was performed by preparative thin-layer chromatography, first with chloroform and then with petroleum ether (30º–65º)-isopropyl ether, 80:20 (ref. 7). After each run the dolichol was extracted from the silica gel with ethyl ether. The purity of the sample was checked by thin-layer chromatography and its identity by its infrared spectrum. Phosphorylation of dolichol was performed using trichloroacetonitrile as condensing agent.[8, 9] After the reaction the mixture was dried *in vacuo*, extracted with chloroform-methanol and washed.

$$\text{UDPG} + \text{acceptor lipid} \rightarrow \text{G-acceptor lipid} + \text{UDP} \qquad (1)$$

$$\text{G-acceptor lipid} + \text{protein} \rightarrow \text{acceptor lipid} + \text{G-protein} \qquad (2)$$

$$\text{G-protein} \rightarrow \text{G} + \text{protein} \qquad (3)$$

1,6 Anhydroglucosan was prepared by heating salicin in 1 *N* NaOH for 10 hr at 100° and neutralizing with Dowex 50 H^+. This preparation contained salicin and 1,6-anhydroclucosan. After chromatography with solvent A (see below), the substances could be visualized with the alkaline silver reagent.[10] Both compounds react slowly. The $R_{glucose}$ of anhydroglucosan varied in different runs between 2.2 and 2.8. Salicin had a 20 % higher mobility.

Chromatography: The following solvents were used for silica gel thin-layer chromatography: (*A*) chloroform-methanol-ammonia-water, 80:30:0.5:3; (*B*) chloroform-methanol-formic acid-water, 70:18.5:8:0.5 (ref. 11); and (*C*) chloroform-methanol-water, 65:25:4. The solvent for paper chromatography was (*D*) *n*-butanol-pyridine-water, 6:4:3. Polyprenols were visualized with the anisaldehyde reagent and lipids with iodine or fluorescein.[7] Localization of the acceptor lipid was performed by removing 1 cm zones from the plates followed by extraction with chloroform-methanol, 2:1, containing 0.6 *N* HCl. The samples were then washed and assayed for acceptor lipid as described below.

Preparation of the enzyme: Rough and smooth rat liver microsomes were prepared according to the method of Moulé *et al.*[12] The smooth fraction was usually more active than the rough but both were used and were very stable if kept frozen with 0.25 *M* sucrose-0.01 *M* glycylglycine. The concentration was adjusted so that 1 ml corresponded to 1 gm of fresh liver.

Assay for lipid glycosylation: A sample of acceptor lipid in chloroform-methanol was mixed with 5 μl of 0.1 *M* Mg-EDTA (prepared by neutralizing ethylenetetraacetic acid with MgO), mixed thoroughly, and dried *in vacuo*. The components of the reaction mixture were then added. The final concentration was: 0.2 *M* glycylglycine, pH 7.5, 0.1 *M* mercaptoethanol, 0.6 % Triton X-100, ^{14}C-UDPG (125,000 counts/min, 205 μc/μmole), and 20 μl of enzyme in a final volume of 50 μl. Before adding the enzyme, the lipid was emulsified with a vortex mixer. After incubation at 37° for 15 min, 0.4 ml of methanol and 0.6 ml of chloroform were added. The protein precipitate was separated by centrifugation and after adding 0.2 ml of 4 mM $MgCl_2$, the chloroform phase was washed as described by Folch *et al.*,[13] dried on planchets, and counted.

Preparation of the acceptor lipid: Ground pig liver was treated with 2 vol of acetone and filtered. The dry residue was extracted with 3 vol of chloroform-methanol, 2:1, and filtered. The extract was made 0.1 *N* in NaOH, and incubated 15 min. at 37°. Sufficient HCl was then added to give a concentration of 0.1 *N* in free acid. After refluxing for 15 min, the chloroform layer was washed according to the technique of Folch *et al.*[13] The extract was then poured into a DEAE cellulose column in the acetate form.[14] The column was washed with chloroform-methanol, 2:1, and then eluted with 0.1 *M* ammonium acetate, pH 4, in chloroform-methanol, 2:1. Fractions which contained acceptor lipid activity were pooled, washed free from ammonium acetate, and concentrated. Further purification was achieved using silica gel thin-layer chromatography with solvent systems A and B in succession. Before preparing the plates, the silica gel was treated with 5 % HCl in ethanol, filtered, and thoroughly washed with ethanol. Acceptor lipid was eluted from the chromatograms with 0.6 *N* HCl in chloroform-methanol, 2:1.

Results. — Glucose transfer to the acceptor lipid: Incubation of the enzyme preparation with UDPG labeled in the glucose moiety and acceptor lipid led to the appearance of radioactivity in the chloroform-soluble fraction. In order to find out if phosphate is also transferred, a parallel incubation with β^{32}P-labeled UDPG was made. The results were as follows:

glucose transferred from ^{14}C-UDPG 3.7 $\mu\mu$mole

phosphate transferred from ^{32}P-UDPG 0.03 $\mu\mu$mole

It was concluded, therefore, that only glucose is transferred.

The conditions for glucolipid formation are shown in Table 1. The reaction is increased by Mg + + but hardly at all by Mn + +. The glucosylation is inhibited completely by EDTA added in excess of Mg + +, and by UDP. Deoxycholate or Triton X-100 usually doubled the yield of glucolipid.

The fact that addition of acceptor lipid gave more chloroform-soluble radioactivity was used for its estimation. As shown in Figure 1, glucosylation was proportional to the amount of acceptor lipid added.

TABLE 1. *Optimal conditions for lipid glucosylation*

Reaction mixture	μμmoles of glucose in chloroform
Complete	9
Minus acceptor lipid	0.75
Minus Mg++	0.08
15 mM Mn++ instead of Mg++	1
Minus deoxycholate	4
Minus mercaptoethanol	7.2
Complete plus 4 mM UDP	0.5

Reaction mixture and assay as described in *Materials and Methods* except that 8.5 mM EDTA was added as sodium salt, Mg ++ was 15 mM, and Triton X-100 was replaced by 0,4 % deoxycholate.

The radioactivity in the lipid increased at first with the time of incubation and decreased subsequently due to its simultaneous disappearance through reaction (2). Therefore accurate measurements of the rate of the glucosylation reaction could not be obtained. Experiments in which the different fractions of liver homogenates were measured showed that the activity was distributed in all the particulate fractions, but the microsomes were always more active. Estimations in the smooth and rough microsome fractions showed no great differences in activity but usually more in the former.

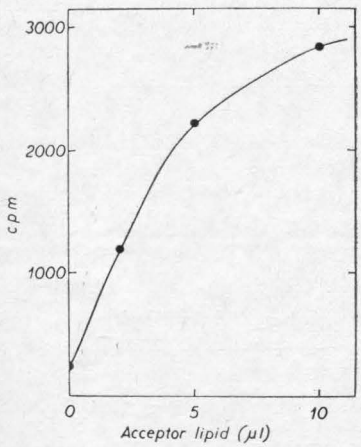

FIG. 1. — Glycosylation as a function of acceptor lipid concentration. Increasing amounts of purified acceptor lipid were added to the reaction mixture and glucosylation was assayed as described in *Materials and Methods*. A purified sample of acceptor lipid (0.53 μmole of phosphate/ml) was used.

Properties and purification of the acceptor lipids The acceptor lipid was found to be soluble in chloroform methanol and in butanol but sparingly soluble in ethyl ether or acetone. The solubility characteristics were those of a phospholipid. Treatment with 1 *N* acid at 100° for six minutes, or 0.05 *N* alkali at 100° for five minutes did not affect acceptor activity. Since it was retained by DEAE-cellulose it was concluded that the acceptor lipid is an acid. Its behavior on thin-layer chromatography agrees with this conclusion. It remained at the origin in alkaline but moved in acid solvents. In solvents A and B the *Rf* was 0 and 0.75, respectively. Acetylation had no effect but methylation with diazo methane destroyed the acceptor lipid activity. Treatment with Br_2 or Fe + + + inactivated the acceptor lipid completely.

Purification of the acceptor lipid could be achieved by decomposition of other lipids with alkali and acid, followed by DEAE-cellulose and thin-layer chromatography. However, considerable losses were often experienced, particularly during thin-layer chromatography presumably due to the presence of Fe +++ in the silica gel. Most of the Fe + + + could be removed by first washing with ethanol-HCl. In this manner considerable purification was obtained (Table 2). The product of the last purification step gave only one spot after thin-layer chromatography with solvents A and B. The purified preparations contained organic phosphate in a form which was only released under drastic conditions.

TABLE 2. *Purification of acceptor lipid.*

	mmoles of organic phosphate	mμmoles of glucose incorporated per μmole of phospate	Recovery %
Crude extract	44.8	0.008	100
After alkaline and acid treatment	4.2	0.06	69
DEADE-cellulose	0.12	1.9	70
Thin-layer chromatography	0.03	6.8	55

Properties of the glucosylated acceptor lipid: The glucosylated acceptor lipid is acid labile. In *n*-propanol at 100° with 5 per cent trichloroacetic acid or in 0.001 *N* HCl, it is completely decomposed in five minutes. Comparable treatment but at pH 6 had no effect. The course of the breakdown at 18° in chloroform methanol containing 0.1 *N* HCl is shown in Figure 2. Under these conditions methyl glucoside is formed whereas in more aqueous solvents glucose is formed as judged by paper chromatography with solvent D. In no case was any galactose detected.

Alkaline treatment (45 min. at 100° with 1 *N* NaOH, in aqueous *n*-propanol) was found to decompose the radioactive glycosylated acceptor lipid so that the radioactivity became water soluble. The compound formed did not migrate in electrophoresis and moved faster than glucose during paper chromatograhy with solvent D. After extracting it from the paper and heating at 100° in 0.5 *N* acid for two hours, it was transformed into a substance which had the mobility of glucose.

Since alkaline degradation in water gave the same compound as in n-propanol it could be excluded that the product formed was a glucoside. Its migration during paper chromatography with solvent D was compared with that of 1,6-anhydroglucosan and it was found that both compounds behaved in the same manner. The rate of formation of 1,6-anhydroglucosan under milder conditions (0.1 *N* NaOH at 64°) is shown in Figure 2.

Transfer from the glucosylated acceptor lipid: Incubation of the glucosylated acceptor lipid with the ensyme preparation at 30° in the presence of 1.3 ,per cent Triton X-100, leads to the changes depicted in Figure 3. It is apparent that the radioactivity which at the start is soluble in chloroform methanol, becomes insoluble, and then gradually becomes water soluble. These reactions do not rerequire Mg^{++}.

The nature of the compound formed first has not been settled definitely but it appears *to be a protein. Treatment with trichloroacetic* acid did not solubilize the radioactive compound. Phenol at 65° extracted most of the radioactivity. Acid hydrolysis (1 *N* SO_4H_2 for 30 min at 100°) led to the liberation of glucose from the compound. Treatment for three hours with 3 *M* KOH at 100° leads to

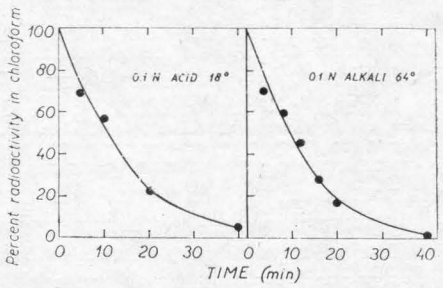

FIG. 2.

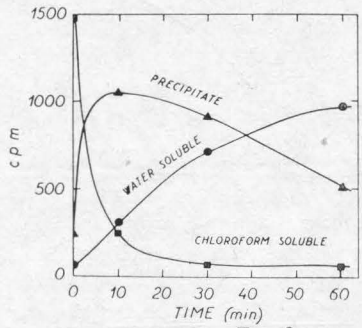

FIG. 3.

FIG. 2. — Acid and alkaline degradation of the glucosylated acceptor lipid. Acid treatment of ^{14}C-glucosyl acceptor lipid: 0.1 *M* HCl in chloroform methanol, 2:1 at 18°. At the indicated times, samples of the reaction mixture were withdrawn, washed free from water soluble products as described under *Materials and Methods,* evaporated in a scintillation vial, and the radioactivity of the remaining glucosyl acceptor lipid measured after dissolving in Bray's solution [15] Alkaline treatment: 0.1 *M* NaOH in *n*-propanol at 64°. Samples were washed after addition of sufficient chloroform to give a twophase system and HCl to make the solution slightly acid.

FIG. 3. — Tranfer from the glucosylated acceptor lipid. Glucosylated acceptor lipid was incubated at 30° with 50 μl of microsomal enzyme and the following reaction mixture: 7mM Na-EDTA, 0.14 *M* glycyglycine, pH 7.5, 0.07 *M* mercaptoethanol, and 1.28% Triton X-100. The reaction was stopped by addition of 1 ml of chloroform methanol, 2:1, and 0.25 ml of 4 mM $MgCl_2$. After centrifugation the precipitate appeared at the chloroform-water interphase. The water was sucked off, the chloroform phase decanted, and the remaining precipitate washed with butanol, transferred to a scintillation vial, and counted with Bray's solution. Both the chloroform and the aqueous phases were counted in a gas flow counter. Counts measured in the scintillator were corrected to the flow-counter efficiency multiplying by 0.75.

the formation of a substance which seemed to be different from the known amino acid-sugar compounds. This point will be studied further.

The reaction in which the radioactivity of the insoluble fraction becomes water soluble (reaction 3) is presumably catalyzed by a glucosidase because the reaction product is mainly glucose.

Synthetic dolichol phosphate: The infrared spectra of purified preparations of acceptor lipid had some similarities with that of dolichol.[5] However, the preparation of pure specimens of acceptor lipid was not easy, so that a short cut was attempted. The unsaponifiable fraction of liver was phosphorylated as described in *Materials and Methods*. The preparation obtained was active in the assay for lipid glucosylation. Phosphorylation of a pure sample of dolichol also gave a product which reacted as acceptor in our test. The compound obtained by the chemical phosphorylation of dolichol is the same as the acceptor isolated from liver as judged by the following criteria (*a*) They both acted as glucose acceptors from UDPG when incubated with the liver enzyme; (*b*) Both resisted acid treatment (5 min in 1 *N* acid at 100°); (*c*) They gave the same R_f on thinlayer chromatography with solvent B ($R_f = 0.81$). Comparison of the two compounds after enzymatic glucosylation showed that (*d*) both released their glucose after five minutes at 100° in 0.01 *N* HCl in 50 per cent *n*-propanol; (*e*) both were decomposed with alkali (1 *N* Na OH in 85 per cent *n*-propanol, 10 min. at 100°) and gave compounds which migrated like 1,6-anhydroglucosan during paper chromatography with solvent D; (*f*) They had the same R_f on thin-laver chromatography with Solvent C (0.20); (*g*) When incubated with the enzyme as described in Figure 3 the synthetic compound behaved the same as the natural compound. That is, the radioactivity was transferred to the insoluble material and appeared afterwards in the aqueous phase.

Discussion. Incubation of the microsomal fraction of liver with UDPG leads to a series of glucose transfer reactions. The first acceptor of glucose is a lipid which has the same properties as the compound obtained by phosphorylation of dolichol. The enzyme trat catalyzes this transfer can use both the natural acceptor lipid and synthetic dolichol phosphate. The glucosylated compound formed from both acceptors appears to be the same as judged by the lability to acid and alkali and by its behavior during thin-layer chromatography. Furthermore, both the natural and synthetic acceptors resist acid treatment under conditions where a pyrophosphate group would be hydrolyzed.

The compounds isolated from bacteria behave in a different manner towards acids. Thus undecaprenol phosphate which is involved in mannan synthesis loses 80 per cent of its phosphate by heating at 100° at pH 2 for 20 minutes.[16] This difference is probably due to the fact that phosphate *in* undecaprenol phosphate is labilized by the allyl structure to which it is bound, whereas in dolichol the first isoprene unit is saturated.

The evidence indicates therefore that the natural acceptor lipid is dolichol monophosphate and following the current nomenclature for nucleotide sugars, the glucosylated compound can be called dolichol monophosphate glucose (DMPG) Many free polyprenols differing in chain length, degree of saturation, and in the number of *cis* and *trans* groups have been isolated from different organisms[17] so that it seems likely that, as in the case of sugar nucleotides, a large family of polyprenol phosphosugars containing various sugar and polyprenol moieties will be found. Furthermore, compounds with either one or two phosphates have already been found, so that the variety of possible intermediates is even larger.

Information on the type of linkage of the glucose in the glucosylated lipid can be deduced from the alkaline degradation. It is known that aryl glycosides yield 1,6-anhydro sugars by alkaline treatment and that enhanced reactivity is found when the OH group at C_2 of the sugar is *trans* to the aglycon.[18] Since the glucosylated acceptor lipid yields 1,6-anhydroglucosan even faster than salicin (β-glucosyl-O-hydroxybenzyl alcohol) it seems likely that it also has a β-linkage.

Data on the nature of the products formed by transfer from the glucosylated acceptor lipid (reactions (2) and (3) are preliminary). One of the few proteins which contain glucose is collagen and transfer from UDPG has been studied.[19, 20] However, in our case, no glucosyl-galactosyl-lysine could be detected as alkaline degradation product of G protein as would be expected if the product of reaction (2) were collagen.

Studies on the nature of the products formed from dolichol monophosphate glucose and similar transfer reactions with other sugars are in progress.

We wish to thank Dr. M. Dankert for having suggested the initial experiments and for his constant advice.

* Suppored in part by grants from the U.S. Public Health Service (GM 03442) and the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

† Career investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

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ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 143, p. 375, 1971

THE ROLE OF DOLICHOL MONOPHOSPHATE IN SUGAR TRANSFER [1-2]

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The specificity of the transfer of monosaccharides from sugar nucleotides to dolichol monophosphate catalyzed by liver microsomes was studied. Besides uridine diphosphate glucose, uridine diphosphate-N-acetylglucosamine and guanosine diphosphate mannose were found to act as donors for the formation of the respective dolichol monophosphate sugars. Uridine diphosphate galacoste and uridine diphosphate-N-acetylgalactosamine gave negative results. The optimal conditions for transfer from dolichol monophosphate glucose to endogenous acceptor was determined. Studies were carried out on the glucosylation of ceramide by brain extracts and of collagen by skin enzymes in order to find out if dolichol monophosphate glucose is an intermediate in these reactions. The results, while not definite were not in favor of this assumption.

A polyprenol phosphate containing eleven isoprene residues has been found to be involved in the synthesis of various cell wall components in bacteria (1–3).

Work with animal tissues (4) has shown that a compound believed to be the monophosphate of dolichol has a role in sugar transfer. Dolichol (5) is a polyprenol containing about twenty isoprene residues, the first of them being saturated. The following reactions were found to be catalyzed by liver microsomes:

$$\text{UDPG} + \text{DMP} \rightarrow \text{DMPG} + \text{UDP}; \quad (1)$$

$$\text{DMPG} + \text{R} \rightarrow \text{DMP} + \text{G-R}; \quad (2)$$

$$\text{G-R} \rightarrow \text{G} + \text{R}.^{4} \quad (3)$$

The identity of DMP was deduced by comparing samples purified from liver with preparations obtained by chemical phosphorylation of purified dolichol. The formation of UDP in Reaction (1) was not proved, but this compound was found to be an inhibitor of DMPG formation. The second step (Reaction 2) could be measured separately from the first and was found to have no specific ion requirements in contrast to the first step (Eq. 1) in which Mg^{2+} ions are necessary.

The nature of the compound represented by G-R in Eq. (2) has not been determined.

There are reports in the literature indicating that lipid intermediates may be involved in the formation of glycoproteins, but no direct transfer from glycolipid to glycoprotein has been reported. Thus Caccam *et al.* (6) and Zatz and Barondes (7) detected the formation of an acid labile mannolipid after incubation of GDP-mannose with various animal enzymes. Similarly Tetas *et al.* (8) described a liver microsome system which catalyzes the synthesis of acid labile lipids of mannose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and galactose. As a continuation of our previous work in this field, several aspects of the problem have been studied further. Tests with several sugar nucleotides with DMP as acceptor and liver microsomes, showed that derivatives of *N*-acetylglucosamine and mannose were formed. Studies were also carried out in order to ascertain the optimal conditions of transfer from DMPG and also, to determine whether it is involved in the glucosylation of ceramide and collagen.

[1] It is a pleasure to dedicate this paper to F. Lynen for his birthday. It is fortunate that DMP sugars have a lipophylic polyprene part which relates them to Dr. Lynen's main interest while it also has a carbohydrate part in keeping with our own field of research. L.F.L.

[2] This investigation was supported in part by a research grant (No. GM 03442) from the National Institutes of Health, U. S. Public Health Service and by the Consejo Nacional de Investigaciones Científicas y Técnicas (R. Argentina).

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[4] Unusual abbreviations: DMP, dolichol monophosphate; DMPG, dolichol monophosphate glucose; DOC, sodium deoxycholate; UDPG, uridine diphosphate glucose.

MATERIALS AND METHODS

Substrates. Radioactive UDPG and UDP-galactose were prepared as described by Wright and Robbins (9). They were separated by paper chromatography in morpholinium borate (10). UDP-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine (both U-^{14}C labelled in the sugar moiety) were purchased from New England Nuclear Corporation. GDP-mannose (U-^{14}C-mannose) was a gift from Dr. Susana Passeron.

Natural DMP was prepared as described previously up to the DEAE-cellulose step (4). Synthetic samples were obtained by chemical phosphorylation of dolichol (4) as described by Popják *et. al.* (11). In order to decompose dolicholpyrophospate, the reaction product was head in n-butanol containing 1 N HCl for 7 min at 100° extracted with chloroform: methanol (2:1) and, washed according to Folch *et al.* (12).

Radioactive DMPG was prepared by incubation of liver microsomes with UDPG-^{14}C and DMP as previously described (4), followed by extraction with chloroform: methanol (2:1). Ceramide-glucon was a gift from Dr. R. Caputto.

Analytical methods. Protein was determined as described by Lowry *et al.* (13) with bovine serum albumin as standard.

Chromatography. The following solvents were used for thin-layer chromatography on silica-gel plates: (A) chloroform: methanol: water (60:20:2; (B) the same as the preceding, but the silica gel was suspended in 1% sodium borate; (C) cloroform:methanol:water (84:15:1); (D) cloroform: methanol:water (65:25:4). For paper chromatography the solvent used was: (E) butanol: pyridine:water (6:4:3) (14).

Enzymes. Liver microsomes were prepared as described in a previous paper (4). The final protein concentration was 30-90 mg/ml. Newborn rat brain microsomes were prepared as described by Basu *et al.* (15) up to the 20.000 g centrifugation step. The pellet from 2 g of brain was resuspended in 0.5 ml of the solution used for homogenization (FIGS. 6 and 7) or in 0.5 ml of 0.1 M glycylglycine buffer, pH 7.8 (Fig. 8). Skin enzyme was prepared by homogenizing the skin from newborn rats in 0.25 M sucrose, 5 mM *EDTA*, and 10 mM 2-mercaptoethanol with a conical glass homogenizer. The homogenate was passed through chessecloth and centrifuged at 100 g for 10 min. The pellet was resuspended in water.

Assay procedures. In the incubation mixtures, DMP or DMPG plus Mg-EDTA, $MgCl_2$, or Na-EDTA were added first; the tubes were dried under reduced pressure, and then the remaining components were added.

RESULTS

Reaction of DMP with various sugar nucleotides. As shown in Table I, incubation of labeled GDP-mannose or UDP-*N*-acetylglucosamine under the conditions previously used for DMPG formation (4), gave rise to the appearance of radioactive lipid. Natural and synthetic DMP were equally effective in increasing the formation of radioactive lipid.

TABLE I

THE FORMATION OF DIFFERENT DMP SUGARS[a]

Nucleotide	Amount (pmoles)	Additions		
		None	Natural DMP (pmoles of sugar transferred)	Synthetic DMP
UDPG	260	3.5	32.0	29.4
UDP-N-acetyl galactosamine	780	2.0	10.8	10.0
GDP-mannose	32.000	20.0	240	243
UDP-N-acetyl galactosamine	780	0.5	0.6	0.6
UDP-galactose	110	0	0	0

a The incubation mixtures contained: 90 mM 2-mercaptoethanol, 0.36 M glycylglycine buffer, pH 7.8, 0.55% Triton X-100, 9 mM Mg-EDTA, 9 mM $MgCl_2$, 20 μl of enzyme, natural or synthetic DMP (containing 13 and 3.4 nmoles of total phosphate, respectively) and radioactive sugar nucleotides in a total volume of 55 μl. After 20 min at 37°, the mixture was processed as previously described for DMPG (4). The specific radioactivity of the sugar nucleotides were (in Ci/mole): UDPG and UDP-galactose, 309; UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine, 43; GDP-mannose, 1.

None was formed from UDP-*N*-acetylgalactosamine or UDP-galactose.

In other experiments UDP-glucuronic acid was tested and found to give some incorporation into lipid with natural DMP but none when synthetic DMP was used as acceptor. ADP-glucose also gave negative results. The fac that DMP stimulates incorporation of sugar into the lipid fraction, suggests that the compounds formed are the DMP derivatives. Furthermore the products obtained from the incubations with GDP-mannose and UDP-*N*-acetylglucosamine with natural DMP gave only one radioactive peak after thin-layer chromatography (solvent D) having the same R_F as DMPG (0.20-0.25).

Properties of the different DMP sugars. The course of decomposition of the different DMP sugars in 0.1 N acid in chloroform: methanol is shown in Fig. 1. The rate is slightly higher for the mannose derivative than for DMPG. The *N*-acetylglucosamine containing compound was considerably more stable. The reason for this difference is difficult to understand. Glucosaminides are known to be more acid-stable than other glycosides, and

this is attributed to the action of the positive $-NH^+$ group in the vicinity of the bond to be hydrolyzed (16). However, when the $-NH_2$ is substituted as in *N*-acetylglucosamines, the rate of hydrolysis is not very different from other glycosides. Comparison of the acid hydrolysis of UDPG and UDP-*N*-acetylglucosamine or glucose 1-phosphate and *N*-acetylglucosamine 1-phosphate showed that the *N*-acetylglucosamine derivatives were about two times more stable, but differences were not as great as those shown in Fig. 1. DMP-*N*-acetylglucosamine was completely decomposed by heating for 10 min at 100° in 0.01 N sulfuric acid in 50 % *n*-propanol. The products obtained were spotted on paper and developed with solvent E. The radioactive peak from

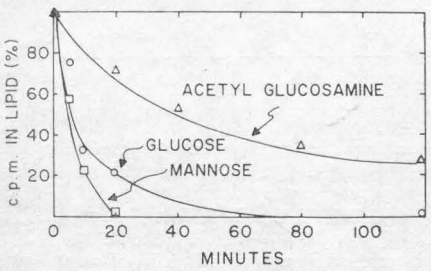

FIG. 1. — Acid treatment of DMP sugars. The DMP sugars were treated with 0.1 N HCl in chloroform: methanol (2:1). After different times at room temperature, the solutions were washed three times with Folch's theoretical upper phase (12) and counted.

hydrolyzed DMP-*N*-acetylglucosamine migrated 20.4 cm while acetylglucosamine migrated 20.3, glucosamine, 12.5, and glucose, 15.2 cm. The product of hydrolysis behaved, therefore, like *N*-acetylglucosamine.

It was reported previously (4) that DMPG is decomposed by alkali giving rise to the formation of 1,6-anhydroglucosan. The mannose and *N*-acetylglucosamine derivates are also decomposed by alkali but slightly more slowly than DMPG. Thus after heating at 100° for 10 min in 0.1 N NaOH, the amount decomposed (%) was as follows: DMPG, 100 per cent; DMP-*N*-acetylglucosamine, 74 per cent; and DMP-mannose, 45 per cent. The fact that the glucose-containing compound is more labile than the others may be understood by comparison with the data on the alkaline decomposition of β-aryl glycosides. The reaction is faster with the β-glucosides, which can form the 1,2-anhydride as intermediate. The formation of this compound requires an OH *trans* to the aglycone so that it is not formed with mannosyl or *N*-acetylglucosaminyl derivates (17). The identity of the products formed by alkaline treatment of the mannose and *N*-acetylglucosamine derivatives has not been investigated yet.

No transfer from DMP-*N*-acetylglucosamine or DMP-mannose could be detected when these compounds were incubated with microsomes under conditions in which DMPG reacted rapidly.

DMPG, DMP-mannose, and DMP-*N*-acetylglucosamine were also formed with the brain microsomal enzymes under the same conditions of Table I.

Conditions for the transfer from DMPG. It was reported previously (4) that transfer from DMPG takes place on incubation of liver microsomes at 30° and with a relatively high detergent concentration, but no detailed study of the optimal conditions had been carried out.

Measurements of the transfer to the insoluble fraction (insoluble in chloroform: methanol, and trichloroacetic acid) at different pH values are shown in Fig. 2. Maximal rate was obtained at pH 7.8 with 50 % values at about 6.3 and 8.3.

The optimal detergent concentration is somewhat variable with the conditions. Dif-

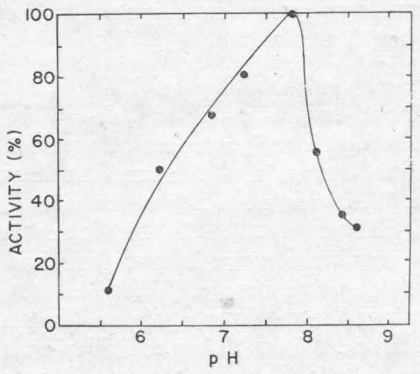

FIG. 2. — Optimal pH for the transfer froh DMPG. The tubes contained: 0.1 M Tris-maleate buffers of different pH, DMPG (4200 cpm, 207 Ci/mole), 8 mM EDTA, 40 mM 2-mercaptoethanol, 0.6% Triton X-100 and 0.47 mg of microsomal proteim in a total volume of 50 μl. After 10 min at 30°, 0.4 ml of methanol and 0.6 ml of chlroform were added. After centrifugation, the precipitate was dried in vacuum, then 1 ml of 5 % trichloroacetic acid was added and the precipitate was washed twice with 1 ml of *n*-butanol, resuspended in Bray solution (24) and counted.

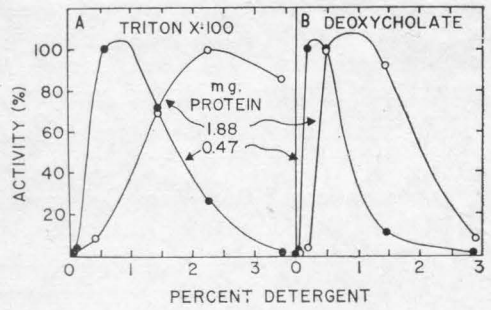

FIG. 3. — Optimal concentration of detergent. The incubation mixtures were the same as in Fig. 2 except that the pH 7.8 buffer and different detergent concentrations were used. Tubes with 0.47 mg of microsomal protein were incubated for 15 min (Fig. 3A) and 12 min (Fig. 3B) at 30° while those with 1.88 mg were incubated 4 and 5 min, respectively. The samples were processed as described in Fig. 2.

ferent optimal values were obtained when different amounts of enzyme were used. As shown in Fig. 3A, the optimal Triton X-100 concentration changes from 0.75 to 2.25 % with a fourfold increase in enzyme. The corresponding values for sodium deoxycholate were 0.3 and 0.90 % (Fig. 3B). Addition of a chloroform: methanol extract of the microsome fraction also produced a displacement of the optimal detergent concentration. The explanation of these changes may be that there are substances that fix detergent thus lowering its effective concentration. No activity was detectable if detergents were replaced by various amounts of organic solvents such as ethanol, *n*-propanol, isopropanol, *n*-butanol, or tert-butyl alcohol.

Time curves with Triton X-100 or DOC at different temperatures are shown in Fig. 4 A, B. At 35° the formation of product ceases after 5-10 min, and the amount decreases thereon. This decrease is due to the liberation of glucose (4). At 25° the formation of product continues for a longer time and reaches a higher maximum. These curves suggested that the enzyme became inactivated during the reaction. This possibility was tested directly by preincubation experiments, the results of which are shown in Fig. 5. In the presence of either DOC or Triton X-100 at 30°, the activity decreases rapidly with a half-time of approximately 9 and 6 min, respectively. No loss of activity occurred on preincubation for 20 min without detergent.

Table II shows the results obtained after incubation under different conditions. It may be observed that the activity obtained with DOC is nearly double that obtained with Triton X-100. These results are similar to those shown in Fig. 4 and 5. Manganese, which is required in many transfer reactions from sugar nucleotides to proteins (6, 18-23) acted as an inhibitor. Magnesium ions were slightly inhibitory, and addition of UDP or UDPG was without effect. No activity was detectable after heating the enzyme for 5 min at 100°.

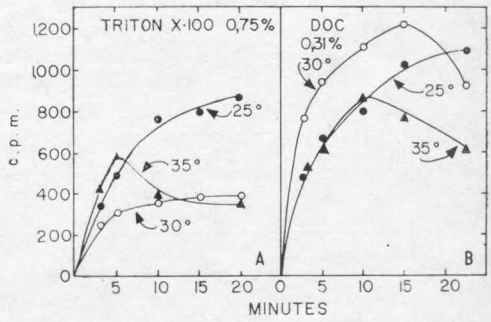

FIG. 4. — Temperature dependence of the glucose transfer to the cloroform:methanol insoluble material. The tubes contained 0.1 M Tris-maleate buffer, pH 7.8, 40 mM 2-mercaptoethanol, 8 mM EDTA, DMPG (33 600 cpm, 207 Ci/mole), 3.76 mg of enzyme protein and 0.75% Triton X-100 (Fig. 4A) or 0.30% DOC (Fig. 4B) in a total volume of 400 µl. Aliquots of 50 µl were taken after different times at the indicated temperatures and processed as described in Fig. 2.

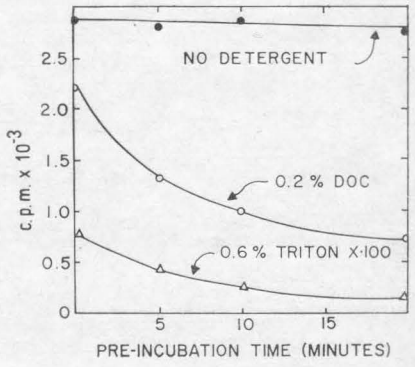

FIG. 5. — Inactivation of the transferring enzymes by detergents. Three different preincubation mixtures were used. The first contained 0.1 M 2-mercaptoethanol, 2.35 mg of microsomal protein and 0.1 M Tris-maleate buffer, pH 7.8, in a total volume of 250 µl. The second and third mixtures contained the same components plus 0.6 % Triton X-100 and 0.2% DOC, respecively. After different preincubation times at 30°, 50-µl aliqunts were taken from the mixtures and transferred to tubes to which DMPG (4200 cpm, 207 Ci/mole) and 0.4 µmole of EDTA had been previously added and dried under reduced pressure. The aliquots from the first incubation mixture were supplemented with DOC to give a final concentration of 0.2%. The tubes were then incubated for 20 min at 23° and processed as described in Fig. 2.

TABLE II

TRANSFER FROM DMPG TO THE CHLOROFORM: METHANOL INSOLUBLE FRACTION[a]

	Final concentration	cpm in precipitate
Complete	—	960
— EDTA	—	880
+ Mn^{2+}	10 mM	270
+ Mg^{2+}	10 mM	550
+ UDP	10 mM	900
+ UDPG	20 mM	925
— Triton X-100 + DOC	0.2%	1940
— Triton X-100 + DOC	0.5%	1580
— Triton X-100 + DOC	1%	470
Heated enzyme (5 min at 100°)		125
Nonincubated		105

[a] The complete system was the same as in Fig. 2 but with the pH 7.8 buffer. The indicated concentrations of Mg^{2+} and Mn^{2+} are the excess over the added EDTA. After 30 min at 30°, the tubes were processed as described in Fig. 2.

Transfer with the skin enzyme. One of the few glycoproteins which contain glucose is collagen. The transfer reaction from UDPG to collagen has been studied by Bosmann and Eylar (18) with a guinea pig fetus skin enzyme and by Spiro and Spiro (19) with kidney glomerular basal membrane. An experiment in which transfer from UDPG to protein and lipid was measured is shown in Table III. Gelatin was used as a protein acceptor. Under the conditions for transfer to collagen (18) that is with Mn^{2+}, radioactivity was incorporated to protein and about fourfold more in the presence of gelatin. DMP produced no change. Under the conditions favorable for DMPG formation no radioactivity was found in protein. DMP increased radioactivity in the chloroform-soluble fraction under both conditions.

Many tests were carried out under varied conditions in order to obtain transfer to protein from DMPG with the skin enzyme. The results were negative. The preliminary conclusion was that DMPG is not an intermediate in the glucosylation of collagen by the skin enzyme. In order to test if the acceptor protein of the liver system is different from collagen two rodioactive proteins were prepared (a) with skin enzyme, gelatin, and UDPG, and (b) with DMPG and liver enzymes. The products were hydrolyzed in 2 N KOH for 20 hr at 100°. Paper electrophoresis at pH 7.8 (0.1 M potassium phosphate) showed that the skin product had a positive charge while the product of the liver enzyme was negatively charged. They were, therefore, clearly different.

TABLE III

Transfer from UDPG with the Skin Enzyme[a]

Conditions	DMP	Gelatin	cpm in protein	cpm in lipid
A. Transfer to DMP	+	+	0	480
	—	+	0	70
	+	—	0	500
	—	—	0	80
B. Transfer to collagen	+	+	4200	480
	—	+	4500	250
	+	—	1200	240
	—	—	1000	140

[a] The incubation mixtures contained: (A) 5 mM Mg-EDTA, 5 mM $MgCl_2$, 0.2 M glycylglycine buffer, pH 7.8, 50 mM 2-mercaptoethanol, 0.3 % Triton X-100, UDPG (150 000 cpm, 309 Ci/mole), natural DRP, 2 mg of gelatin and 50 µl of skin enzyme in a total volume of 100 µl; (B) 20 mM $MnCl_2$, 0.01% Triton X-100, 0.1 M acetate buffer, pH 5.8, UDPG (150 000 cpm, 309 Ci/mole), natural DMP, 2 mg of gelatin and 50 µl of skin enzyme in a total volume of 100 µl. After 30 min at 37°, the reactions were stopped by the addition of 0.4 ml methanol plus 0.6 ml of chloroform. The soluble fractions were washed according to Folch *et al.* (12) and counted. The insoluble fractions were washed twice with 0.5 ml of 0.1 % phosphotungstic acid in 0.5 N HCl, twice with 0.5 ml of 66 % ethanol and counted.

Brain enzymes. The microsomal fraction from chick embryo brain catalyzes the transfer of glucose from UDPG to ceramide (15). Experiments were carried out with a similar enzyme preparation from newborn rats in order to find out if DMPG is an intermediate in this process.

Ceramide-glucose is relatively acid-stable while DMPG is completely hydrolyzed in 45 min at room temperature in choroform: methanol (2:1) containing 0.1 N HCl so that the two compounds can be easily distinguished. The result of incubating the brain enzyme with radioactive UDPG in the presence of DMP and Mg^{2+} is shown in Fig. 6A. A considerable amount of radioactivity appeared in the chloroform: methanol soluble fraction. Both acid-stable and labile compounds were

formed, and after 45 min there was about 50 % of each.

Magnesium ions are required for the transfer from UDPG to DMP with the liver enzyme (4). On the other hand, transfer to ceramide has been reported to have no specific cation requirements (15). As shown in Fig. 6B, in the presence of excess EDTA, the formation of the acid-labile compound was completely inhibited, while the synthesis of the acid-resistant one was hardly affected. Under the conditions of Fig. 6B, exogenously added DMPG was only slightly metabolized, but no chloroform: methanol soluble acid-stable material was formed.

Under the conditions of Fig. 6A, the presence of DMP greatly stimulated the formation of the acid-labile compound while that of the stable one was slightly diminished (Fig. 7). This inhibition of the acid-resistant incorporation is possibly due to some substance in the DMP solution and not to a competition between both substrates (DMP and ceramide) for UDPG, because the same inhibition occurred in the conditions of Fig. 6B, that is in the presence of excess EDTA where no incorporation to the acid-labile compound was detectable.

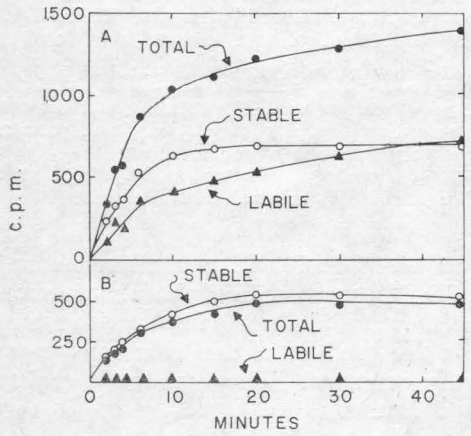

FIG. 6. — Glucose transfer to a chloroform: methanol soluble material by brain microsomes. The incubation mixtures contained: (A) Synthetic DMP, 8 mM $MgCl_2$, 8 mM Mg-EDTA, 0.5 % Triton X-100, 0.33 M glycylglycine buffer, pH 7.8, 250 µl of enzyme and 20 nmoles of UDPG (207 Ci/mole) in a total volume of 600 µl. (B) The same components except that instead of $MgCl_2$ and Mg-EDTA the mixture contained 33 mM Na-EDTA. Aliquots of 50 µl were taken after different times at 37° and placed in tubes containing 0.5 ml of chloroform: methanol (2:1) plus 120 µl of a 42 mM KCl, 21 mM EDTA solution. The lower phase was washed three times with 200 µl of chloroform: methanol: 0.1 M KCl (3:47:48) and carefully poured off to other tubes in order to separate the precipitates. The lower phase was then divided in two halves, *a* for total and *b* for acid-stable radioactivity. Sample *b* was made up to 1 ml with chloroform: methanol (2:1) and 20 µl of 5 N HCl were added. After 45 min at room temperature, the sample was washed twice as described above. The radioactivity *a-b* was taken as that of acid-labile compounds.

The identity of the compounds formed was established as follows. Thin-layer chromatography (solvent D) of the product obtained under the conditions described in Fig. 6A in which both acid-labile and stable compounds were formed by the brain enzyme, gave two peaks of radioactivity (R_F = 0.63 and 0.2). If the samples were treated with mild acid before chromatography, only the fast peak appeared. This substance seemed to be ceramide-glucose since its mobility in three different solvents (A, B and C) was the same as that of a ceramide-glucose standard and because it gave glucose by heating with 2 N H_2SO_4 for 4 hr at 100° (identified with solvent E). The acid-stable compound formed in the presence or absence of Mg^{2+} or of DMP seemed to be the same as judged by the above mentioned criteria.

As to the slow moving, acid-labile substance, its properties are those of DMPG. Its mobility during thin-layer chromatography was the same (solvent D, R_F: 0.2). As a further criterion of identity, the substance was treated with alkali, and it was found that 1,6-anhy-

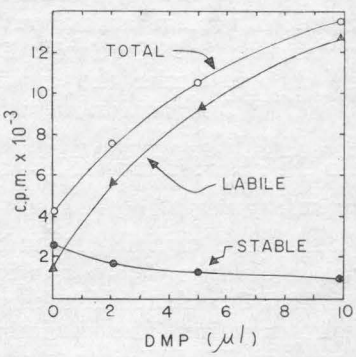

FIG. 7. — Stimulation by DMP of the incorporation to the chloroform: methanol soluble acid-labile fraction. The incubation mixtures contained: 8 mM $MgCl_2$, 8 mM Mg-EDTA, 25 µl of enzyme, 0.5 % Triton X-100, 0.33 M glycylglycine buffer, pH 7.8, 4 nmoles of UDPG (207 Ci/mole) and different amounts of synthetic DMP in a total volume of 60 µl. After 60 min at 37° the tubes were processed as described for the 50 µl aliquots in Fig. 6.

droglucosan was formed (identified with solvent E as previously described, Ref. 4). The acid-labile product formed in the presence or absence of DMP appeared to be the same as judged by its mobility during thin-layer chromatography and by the formation of 1,6-anhydroglucosan by alkaline treatment.

The results represented in Fig. 6 A, B show that the glucosylation of ceramide proceeds in the presence of EDTA while the formation of DMPG is inhibited so that it seems unlikely that the latter is a glucose donor for ceramide-glucose formation.

The brain extract was also examined for the presence of the enzyme which catalyzes the transfer from DMPG to acceptor. As shown in Fig. 8, the changes were the same as those reported before for the liver enzyme (4). The radioactivity which is initially all soluble in chloroform: methanol (2:1) was transferred to the protein fraction and afterwards appeared in the aqueous phase. The water soluble radioactivity was identified as glucose by paper chromatography with solvent E. No chloroform: methanol-soluble, acid-stable material was formed. In the same conditions ceramide-glucose was not metabolized. It seems therefore that the metabolism of DMPG is the same in brain and in liver.

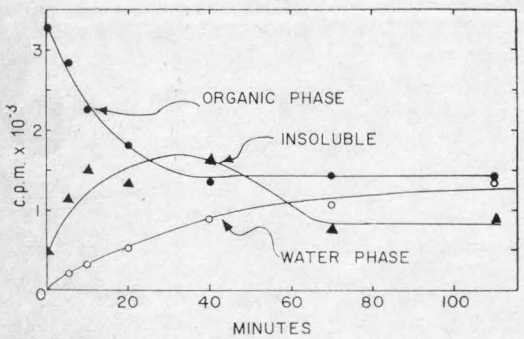

FIG. 8. — The metabolism of DMPG by brain microsomes. The incubation mixture contained: 7 mM EDTA, 1.6 % Triton X-100, 0.13 M glycylglycine buffer, pH 7.8 60 mM 2-mercaptoethanol, DMPG (38 000 cpm, 309 Ci/mole) and 600 µl of enzyme in a total volume of 835 µl. Aliquots of 100 µl were taken of different times at 30°. They were processed as described in Fig. 6 for the 50 µl aliquots except that the 0.5 ml of chloroform: methanol (2:1) contained 20 µl of a 0.25 M *KCl*-0125 M EDTA solution. The protein fraction was washed twice with 1 ml of *n*-butanol, resuspended in Bray solution (24) and counted.

DISCUSSION

According to the results reported in this paper, DMP sugars containing *N*-acetylglucosamine or mannose are formed from the respective nucleotides. These compounds are acid-labile like the glucose compound, but DMP-*N*-acetylglucosamine is more stable than the glucose derivative. This is a rather unexpected property since according to the data in the literature the rate of hydrolysis of β-methylglucoside is 9.3 times slower than that of β-methyl *N*-acetylglucosaminide. The relation is similar for the α glucosides (16). It is known that glucosaminides are more stable to acid presumably due to the effect of the positive charge on the amino group. However, acid hydrolysis of DNP-*N*-acetylglucosamine gave *N*-acetylglucosamine and not glucosamine.

As to the utilization of DMP-*N*-acetylglucosamine and mannose, nothing is known as yet. The reaction from DMPG to acceptor is catalyzed by the microsome fraction of liver and brain, but this does not occur with the other two compounds. Probably it is only a question of finding the appropriate conditions.

The other question which required investigation was whether DMPG is involved in other transfer reactions. For instance in the glucosylation of collagen, ceramide, and glycogen. In these cases the available evidence is against the participation of DMPG.

In the glucosylation of collagen catalyzed by skin enzymes with UDPG as donor, it was found that there was no relation between transfer to DMP and to the protein acceptor. The results were not in favor of DMPG as intermediate.

The transfer from UDPG to endogenous ceramide to give ceramide-glucose is catalyzed by a brain enzyme. Addition of DMP increased DMPG but not the rate of ceramide-glucose formation. Excess EDTA inhibited transfer to DMP but not to ceramide. This seems to be a fairly strong evidence in favor of the nonparticipation of DMPG in ceramide-glucose formation. Similarly, transfer from UDPG to glycogen can take place with EDTA concentrations which inhibit completely DMPG formation (25). Here again the evidence is against the intermediate formation of DMPG.

The role of DMP sugars as intermediates, therefore, appears to be restricted to some reactions, the significance of which is not known yet.

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Este libro se terminó de imprimir en los Talleres Gráficos A. Baiocco S.R.L. el día 14 de diciembre de 1973 siendo su tirada de 1.000 ejemplares. Estuvo al cuidado de la obra el ex Presidente de la Academia de Ciencias Exactas, Físicas y Naturales, Ing. ERNESTO E. GALLONI.